

REPORT DOCUMENTATION PAGE			Form Approved OMB NO. 0704-0188		
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1. REPORT DATE (DD-MM-YYYY) 07-01-2013		2. REPORT TYPE Final Report		3. DATES COVERED (From - To) 1-Jul-2007 - 30-Dec-2012	
4. TITLE AND SUBTITLE Physiological Feedback Control 2011-2012 Annual Report			5a. CONTRACT NUMBER W911NF-07-1-0437		
			5b. GRANT NUMBER		
			5c. PROGRAM ELEMENT NUMBER 7620AI		
6. AUTHORS Thommey Thomas, James R. Baker Jr., Abraham Van Der Spek, Seok Ki Choi, Pascale Leroueil, Clara Verweij, Baohua Huang, Brent Ward			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAMES AND ADDRESSES University of Michigan - Ann Arbor Regents of the University of Michigan 3003 S. State St Ann Arbor, MI 48109 -1274				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Research Office P.O. Box 12211 Research Triangle Park, NC 27709-2211				10. SPONSOR/MONITOR'S ACRONYM(S) ARO	
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13. SUPPLEMENTARY NOTES The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.					
14. ABSTRACT The research completed within this contract focused on the development of a platform that reverses the effects of an analgesic overdose (Tasks 1-3), and the development a platform that reverses the effects associated with organophosphate poisoning (Task 4). We successfully scaled up the synthesis of the naloxone prodrug that was designed and tested during Phases I and					
15. SUBJECT TERMS naloxone prodrug, hypoxia-induced release of naloxone, rat model, pig model, extend the effective half-life of Pralidoxime, plasmin-sensitive hydrogel, hemostatic agent					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON James Baker
a. REPORT UU	b. ABSTRACT UU	c. THIS PAGE UU			19b. TELEPHONE NUMBER 734-647-2777

Report Title

Physiological Feedback Control 2011-2012 Annual Report

ABSTRACT

The research completed within this contract focused on the development of a platform that reverses the effects of an analgesic overdose (Tasks 1-3), and the development a platform that reverses the effects associated with organophosphate poisoning (Task 4).

We successfully scaled up the synthesis of the naloxone prodrug that was designed and tested during Phases I and II, and documented its efficacy in a pig model. Although we had proposed to develop a long-acting form of ketamine, regulatory issues prevented us from moving that project forward. Finally, we designed a novel class of dendrimer-oxime drug conjugates, and evaluated the mechanism by which these conjugates hydrolyze paraoxon.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received Paper

- 08/28/2012 16.00 Michael Wallisch, Nehad M. El Rody, Baohua Huang, Dennis R. Koop, James R. Baker, George D. Olsen. Naloxone pro-drug rescues morphine induced respiratory depression in Sprague-Dawley rats, *Respiratory Physiology & neurobiology*, (01 2012): 52. doi: 10.1016/j.resp.2011.10.009
- 08/28/2012 17.00 Baohua Huang , Jolanta F. Kukowska-Latallo, Shengzhuang Tang, Hong Zong , Kali B. Johnson, Ankur Desai, Chris L. Gordon , Pascale R. Leroueil, James R. Baker Jr.. The facile synthesis of multifunctional PAMAM dendrimer conjugates through copper-free click chemistry, *Bioorganic & Medicinal Chemistry Letters*, (03 2012): 3152. doi:
- 08/28/2012 14.00 Baohua Huang, Shengzhuang Tang, Ankur Desai, Kyung-Hoon Lee, Pascale R. Leroueil,, James R. Baker Jr. . Novel Poly(EThyleneAmidoAmine) (PETAA) dendrimers produced through a unique and highly efficient synthesis, *Polymer*, (11 2011): 5975. doi:
- 08/30/2011 7.00 Thommey P. Thomas, Baohua Huang, Ankur Desai, Hong Zong, Xue-min Cheng, Alina Kotlyar, Pascale R. Leroueil, Thomas Dunham, Abraham van der Spek, Brent B. Ward, James R. Baker. Plasma-mediated release of morphine from synthesized prodrugs, *Bioorganic & Medicinal Chemistry Letters*, (11 2010): 0. doi: 10.1016/j.bmcl.2010.08.098
- 08/30/2011 8.00 Daniel Q. McNerny, Pascale R. Leroueil, James R. Baker. Understanding specific and nonspecific toxicities: a requirement for the development of dendrimer-based pharmaceuticals, *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, (05 2010): 0. doi: 10.1002/wnan.79
- 08/30/2011 9.00 Christopher V. Kelly, Thommey P. Thomas, Istvan Majoros, Bradford G. Orr, James R. Baker, Mark M. Banaszak Holl, Douglas G. Mullen, Daniel Q. McNerny, Ankur Desai, Xue-min Cheng, Stassi C. DiMaggio, Alina Kotlyar, Yueyang Zhong, Suyang Qin. Design, Synthesis, and Biological Functionality of a Dendrimer-Based Modular Drug Delivery Platform, *Bioconjugate Chemistry*, (04 2011): 0. doi: 10.1021/bc100360v
- 08/30/2011 10.00 Mark M. Banaszak Holl, James R. Baker, Jr, Douglas G. Mullen, Ankur M. Desai, Mark Andreae. Acetonitrile shortage: Use of isopropanol as an alternative elution system for ultra/high performance liquid chromatography, *Analytical Methods*, (01 2011): 0. doi: 10.1039/c0ay00493f
- 08/30/2011 11.00 Douglas G. Mullen, Mark M. Banaszak Holl. Heterogeneous Ligand–Nanoparticle Distributions: A Major Obstacle to Scientific Understanding and Commercial Translation, *Accounts of Chemical Research*, (08 2011): 0. doi: 10.1021/ar1001389
- 08/30/2011 12.00 Seok Ki Choi, Pascale Leroueil, Ming-Hsin Li, Ankur Desai, Hong Zong, Abraham F. L. Van Der Spek, James R. Baker. Specificity and Negative Cooperativity in Dendrimer–Oxime Drug Complexation, *Macromolecules*, (06 2011): 0. doi: 10.1021/ma200522m
- 11/02/2012 22.00 Thommey P. Thomas, Baohua Huang, Seok Ki Choi, Justin E. Silpe, Alina Kotlyar, Ankur M. Desai, Hong Zong, Jeremy Gam, Melvin Joice, James R. Baker. Polyvalent Dendrimer-Methotrexate as a Folate Receptor-Targeted Cancer Therapeutic, *Molecular Pharmaceutics*, (09 2012): 0. doi: 10.1021/mp3002232
- 11/02/2012 21.00 Jolanta_Kukowska-Latallo , Shengzhuang_Tang , Hong_Zong, Kali_B_Johnson , Ankur_Desai , Chris_L_Gordon, Baohua_Huang, Pascale_R_Leroueil, James_R_Baker_JR. The facile synthesis of multifunctional PAMAM dendrimer conjugates through copper-free click chemistry, *Bioorganic & Medicinal Chemistry Letters*, (05 2012): 3152. doi:
- 11/02/2012 23.00 Hong_Zong, Thommey_P_Thomas,, Kyung-Hoon_Lee, Ankur_M_Desai, Ming-hsin-Li, Alina_Kotlyar, Yuehua_Zhang, Pascale_R_Leroueil, Jeremy_J_-Gam, Mark_M_Banaszak_Holl, James_R_Baker_Jr.. Bifunctional PAMAM dendrimer conjugates of folic acid and methotrexate with defined ratio., *Biomacromolecules*, (02 2012): 982. doi:

11/02/2012	24.00	Seok Ki Choi, Thommey P. Thomas, Ming-Hsin Li, Ankur Desai, Alina Kotlyar, James R. Baker. Photochemical release of methotrexate from folate receptor-targeting PAMAM dendrimer nanoconjugate, Photochemical and Photobiological Sciences, (01 2012): 653. doi: 10.1039/c2pp05355a
11/02/2012	26.00	Ming-Hsin_Li, Seok_Ki_Choi, Thommey_P_Thomas, Ankur_Desai, Kyung-Hoon_Lee, Alina_Kotlyar, Mark_M_Banaszak_Holl, James_R_Baker. Dendrimer-based multivalent methotrexates as dual acting nanoconjugates for cancer cell targeting, European Journal of Medicinal chemistry, (01 2012): 560. doi: 10.1016/j.ejmech.2011.11.027
11/02/2012	27.00	Michael_Wallisch , Nehad_M_El_Rody, Baohua_Huang, Dennis_R_Koop, James_R_Baker_Jr., George_D_Olsen. Naloxone pro-drug rescues morphine induced respiratory depression in Sprague-Dawley rats, Respiratory Physiology & Neurobiology, (01 2012): 52. doi:
11/02/2012	28.00	Brent_B_Ward, Thomas_Dunham, Istvan_J_Majoros, James_R_Baker. Targeted Dendrimer Chemotherapy in an Animal Model for Head and Neck Squamous Cell Carcinoma, J Oral Maxillofacial Surgery, (09 2011): 2452. doi: 10.1016/j.joms.2010.12.041

TOTAL: 16

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

<u>Received</u>	<u>Paper</u>
11/02/2012	19.00 Seok_Choi , Thommey_P_Thomas, Pascale_Leroueil, Alina_Kotlyar, Abraham_F_Van_Der_Spek, James_R_Baker_JR. Specific and Cooperative Interactions between Oximes and PAMAM Dendrimers As Demonstrated by (1)H NMR Study, Journal of Physical Chemistry B, (08 2012): 103. doi:
11/02/2012	25.00 Seok-Ki-Choi, Manisha_Verma, Justin_Silpe, Ryan_E_Moody, Kenny_Tang, Jeffrey_J_Hanson, James_R_Baker_Jr.. A photochemical approach for controlled drug release in targeted drug delivery, Bioorganic & Medicinal Chemistry, (12 2011): 1281. doi:

TOTAL: 2

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Huang B, Thomas T, Desai A, Tang S, Leroueil P, Baker JR Jr. The development of dendrimer platforms for targeted drug delivery via combinatorial copper-free click chemistry. Poster, Department of Internal Medicine 19th Annual Research Day, University of Michigan, Ann Arbor MI May 20, 2011.

Number of Presentations: 1.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received

Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received

Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

<u>Received</u>	<u>Paper</u>
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04/01/2010	1.00	Baohua Huang, Shengzhuang Tang, Ankur Desai, Xue-min Cheng, Alina Kotlyar, Abraham Van Der Spek, Thommey P. Thomas, James R. Baker Jr.. Human plasma-mediated hypoxic activation of indolequinone-based naloxone pro-drugs, Bioorganic & Medicinal Chemistry Letters (07 2009)
04/01/2010	2.00	Douglas G. Mullen, Emilee L. Byrne, Ankur M. Desai, Mallory A. van Dongen, Mark Barash, Xue-min Cheng, James R. Baker Jr. and Mark M. Banaszak Holl. Isolation and characterization of dendrimer with precise numbers of functional groups, Nanotechnology (04 2010)
04/15/2010	4.00	Ankur M. Desai, Mark Andreae, Douglas G. Mullen, Mark M. Banaszak-Holl, and James R. Baker Jr.. Acetonitrile Shortage: Use of Isopropanol as an alternative elution system for Ultra/High Performance Liquid Chromatography, The Analyst (04 2010)
04/26/2010	5.00	Douglas G. Mullen, Emilee L. Byrne, Ming Fang, Daniel Q. McNerny, Ankur Desai, James R. Baker Jr., Bradford G. Orr, Mark M. Banaszak Holl. The effect of mass transport in the synthesis of partially acetylated dendrimer: Implications for functional ligand-nanoparticle distributions, Macromolecules (04 2010)
06/12/2012	13.00	Choi, Seok Ki, Thomas, Thommey P , Leroueil, Pascale, Kotlyar, Alina, Van Der Spek, Abraham F L, Baker, Jr., James R. Specific and cooperative interactions between oximes and PAMAM dendrimers as demonstrated by 1H NMR study, J Phys Chem (06 2012)
08/28/2012	15.00	Seok Ki Choi, Thommey P. Thomas, Pascale Leroueil, Alina Kotlyar, Abraham F. L. Van Der Spek, James R. Baker, Jr.. Specific and Cooperative Interactions between Oximes and PAMAM2 Dendrimers As Demonstrated by 1H NMR Study, The Journal of Physical Chemistry B (submitted) (08 2012)

TOTAL: 6

Number of Manuscripts:

Books

<u>Received</u>	<u>Paper</u>
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TOTAL:

Patents Submitted

Invention Title: UM 3709 – Dendrimeric Prodrug as a Controlled Release Formulation in Pain Management – Patent Title: Dendrimer Conjugates
Patent/Application Numbers: 61/101,461; 12/570,977
Is the Patent Filed in the US?: Yes
Is the Patent Filed in a Foreign Country?: No
Was the confirmatory instrument or assignment forwarded to the contracting officer?: Yes
Foreign Countries of application: None

Invention Title: UM 4538 Synthesis of Baker-Huang PAMAM Dendrimers; Patent Title Dendrimer Compositions and Methods of Synthesis
Patent/Application Numbers: 61/251,244 and 13/502,004 (US), 2010318637 (AU), PCT/US2010/051835 (CN), 2777682 (CA)
Is the Patent Filed in the US?: Yes
Is the Patent Filed in a Foreign Country?: Yes
Was the confirmatory instrument or assignment forwarded to the contracting officer?: Yes
Foreign Countries of application: China, Canada, Australia

Invention Title: UM 4603 – Block Synthetic Method for Dendrimer Synthesis
Patent/Application Numbers: Not filed
Is the Patent Filed in the US?: No
Is the Patent Filed in a Foreign Country?: No
Was the confirmatory instrument or assignment forwarded to the contracting officer?: Yes
Foreign Countries of application: No

Invention Title: UM 4798 – Multifunctional Small Molecules
Patent/Application Numbers: 13/504,046
Is the Patent Filed in the US?: Yes
Is the Patent Filed in a Foreign Country?: No
Was the confirmatory instrument or assignment forwarded to the contracting officer?: Yes
Foreign Countries of application: No

Invention Title: UM 4901 – Dual Function Real-Time Sensor
Patent/Application Numbers: Per legal counsel not filed
Is the Patent Filed in the US?: No
Is the Patent Filed in a Foreign Country?: No
Was the confirmatory instrument or assignment forwarded to the contracting officer?: Yes
Foreign Countries of application: None

Invention Title: UM 4902 – Bioorthogonal Nanoparticle Reporter Alkyne System
Patent/Application Numbers: 61/562,767
Is the Patent Filed in the US?: Yes; Synthesizing Functionalized Dendrimers within Biological Settings
Is the Patent Filed in a Foreign Country?: No
Was the confirmatory instrument or assignment forwarded to the contracting officer?: Yes
Foreign Countries of application: None

Invention Title: UM 4966 – Plasmin Release
Patent/Application Numbers: None
Is the Patent Filed in the US?: No
Is the Patent Filed in a Foreign Country?: No
Was the confirmatory instrument or assignment forwarded to the contracting officer?: Yes
Foreign Countries of application: None

Invention Title: UM 5132 – Dendrimer Conjugate and Methotrexate Analogues
Patent/Application Numbers: 61/568,521(provisional) – Multifunctional Small molecules
Is the Patent Filed in the US?: Yes
Is the Patent Filed in a Foreign Country?: No
Was the confirmatory instrument or assignment forwarded to the contracting officer?: Yes
Foreign Countries of application: None

Invention Title: UM 5186 – Organophosphate Antidotes
Patent/Application Numbers: 61/625,911(provisional) – Multifunctional Small molecules
Is the Patent Filed in the US?: Yes
Is the Patent Filed in a Foreign Country?: No
Was the confirmatory instrument or assignment forwarded to the contracting officer?: Yes
Foreign Countries of application: Not determined at this point in time

Invention Title: UM 5217 – Antibacterial Dendrimer
Patent/Application Numbers: Not filed
Is the Patent Filed in the US?:
Is the Patent Filed in a Foreign Country?:
Was the confirmatory instrument or assignment forwarded to the contracting officer?: Yes
Foreign Countries of application: No

Invention Title: UM 5608 – Fluorescent Dye Conjugate
Patent/Application Numbers: Not filed
Is the Patent Filed in the US?:
Is the Patent Filed in a Foreign Country?:
Was the confirmatory instrument or assignment forwarded to the contracting officer?: Yes
Foreign Countries of application: No

Patents Awarded

Invention Title: UM 3709 – Dendrimeric Prodrug as a Controlled Release Formulation in Pain Management – Patent Title:
~~Dendrimer Conjugates~~
Patent/Application Numbers: 61/101,461; 12/570,977
Is the Patent Filed in the US?: Yes
Is the Patent Filed in a Foreign Country?: No
Was the confirmatory instrument or assignment forwarded to the contracting officer?: Yes
Foreign Countries of application: None

Invention Title: UM 4538 Synthesis of Baker-Huang PAMAM Dendrimers; Patent Title Dendrimer Compositions and Methods of Synthesis
Patent/Application Numbers: 61/251,244 and 13/502,004 (US), 2010318637 (AU), PCT/US2010/051835 (CN), 2777682 (CA)
Is the Patent Filed in the US?: Yes
Is the Patent Filed in a Foreign Country?: Yes
Was the confirmatory instrument or assignment forwarded to the contracting officer?: Yes
Foreign Countries of application: China, Canada, Australia

Invention Title: UM 4798 – Multifunctional Small Molecules
Patent/Application Numbers: 13/504,046
Is the Patent Filed in the US?: Yes
Is the Patent Filed in a Foreign Country?: No
Was the confirmatory instrument or assignment forwarded to the contracting officer?: Yes
Foreign Countries of application: No

Awards

Graduate Students

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	National Academy Member
Baohua Huang, Ph.D.	0.50	
James R. Baker Jr., M.D. - PI	0.80	
Seok Ki Choi, Ph.D.	0.45	
Thommey P. Thomas, Ph.D.	0.50	
Abraham Van Der Spek, M.D.	0.10	
Brent Ward, M.D.	0.30	
Hong Zong, Ph.D.	0.45	
FTE Equivalent:	3.10	
Total Number:	7	

Names of Under Graduate students supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	Discipline
Melvin Joice	0.00	
James Otis	0.00	
Olga Lykhytska	0.00	
Steven Walker	0.00	
Mark Andreae	0.00	
Veronica Choe	0.00	
Yang Chen	0.00	
Matthew Vosters	0.00	
FTE Equivalent:	0.00	
Total Number:	8	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	1.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	1.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	1.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):.....	1.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PhDs

NAME

Total Number:

Names of other research staff

NAME

PERCENT SUPPORTED

Mark Vartanian, B.S. 1.00

Pascale Leroueil, Ph.D. 0.10

Wen-Xiang Zhang, M.D. 0.25

Shengzhuang Tang, M.S. 1.00

Ankur Desai, M.S. 0.25

Alina Kotlyar, M.S. 1.00

FTE Equivalent: 3.60

Total Number: 6

Sub Contractors (DD882)

Inventions (DD882)

5 **Plasmin Release**

Patent Filed in US? (5d-1) N

Patent Filed in Foreign Countries? (5d-2) N

Was the assignment forwarded to the contracting officer? (5e) Y

Foreign Countries of application (5g-2):

5a: James R. Baker Jr

5f-1a: Univ of Michigan

5f-c: 1150 W Medical Ctr Dr

Ann Arbor MI 48109

5a: Baohua Huang

5f-1a: Univ of Michigan

5f-c: 1150 W Medical Ctr Dr

Ann Arbor MI 48109

5a: Pascale R. Leroueil

5f-1a: Univ. of Michigan

5f-c: 1150 W Medical Ctr Dr

Ann Arbor MI 48109

5 **UM 3709 – Dendrimeric Prodrug as a Controlled Release Formulation in Pain Management – Patent Title: Dendrimer Conjugates**

Patent Filed in US? (5d-1) Y

Patent Filed in Foreign Countries? (5d-2) N

Was the assignment forwarded to the contracting officer? (5e) Y

Foreign Countries of application (5g-2):

5a: Abraham F. L. Van Der Spek

5f-1a: University of Michigan

5f-c: 1150 W. Medical Ctr Dr

Ann Arbor MI 48109

5a: James R. Baker Jr

5f-1a: Univeristy of Michigan

5f-c: 1150 W. Medical Ctr Drive

Ann Arbor MI 48109

5a: Xue-min Cheng

5f-1a: Univ. of Michigan

5f-c: 1150 W Medical Ctr Drive

Ann Arbor MI 48109

5 **UM 4538 Dendrimer Compositions and Methods of Synthesis**

Patent Filed in US? (5d-1) Y
Patent Filed in Foreign Countries? (5d-2) Y
Was the assignment forwarded to the contracting officer? (5e) Y
Foreign Countries of application (5g-2): AU, CN, CA

5a: James R Baker Jr

5f-1a: Univ of Michigan

5f-c: 1150 W Medical Ctr Dr
Ann Arbor MI 48109

5a: Baohua Huang

5f-1a: Univ of Michigan

5f-c: 1150 W Medical Ctr Dr
Ann Arbor MI 48109

5 **UM 4603 – Block Synthetic Method for Dendrimer Synthesis**

Patent Filed in US? (5d-1) N
Patent Filed in Foreign Countries? (5d-2) N
Was the assignment forwarded to the contracting officer? (5e) N
Foreign Countries of application (5g-2): none

5a: James R. Baker Jr.

5f-1a: Univ of Michigan

5f-c: 1150 W Medical Ctr Dr
Ann Arbor MI 48109

5a: Yuehua Zhang

5f-1a: Univ of Michigan

5f-c: 1150 W Medical Ctr Dr
Ann Arbor MI 48109

5 **UM 4798 - Multifunctional Small Moelcules**

Patent Filed in US? (5d-1) Y
Patent Filed in Foreign Countries? (5d-2) N
Was the assignment forwarded to the contracting officer? (5e) Y
Foreign Countries of application (5g-2):

5a: James R. Baker Jr

5f-1a: Univ of Michigan

5f-c: 1150 W Medical Ctr Dr
Ann Arbor MI 48109

5a: Hong Zong

5f-1a: Univ of Michigan

5f-c: 1150 W Medical Ctr Dr
Ann Arbor MI 48109

5a: Thommey P Thomas

5f-1a: Univ of Michigan

5f-c: 1150 West Med Ctr Dr

Ann Arbor MI 48109

5 UM 4901 – Dual Function Real-Time Sensor

Patent Filed in US? (5d-1) N

Patent Filed in Foreign Countries? (5d-2) N

Was the assignment forwarded to the contracting officer? (5e) N

Foreign Countries of application (5g-2):

5a: Sascha N. Goonewardena

5f-1a: Univ of Michigan

5f-c: 1150 W Medical Ctr Dr

Ann Arbor MI 48109

5a: James R. Baker Jr

5f-1a: Univ of Michigan

5f-c: 1150 W Medical Ctr Dr

Ann Arbor MI 48109

5a: Hong Zong

5f-1a: Univ of Michigan

5f-c: 1150 W Medical ctr Dr

Ann Arbor MI 48109

5a: Hong Zong

5f-1a: Univ of Michigan

5f-c: 1150 W Medical Ctr Dr

Ann Arbor MI 48109

5 UM 4902 – Bioorthogonal Nanoparticle Reporter Alkyne System

Patent Filed in US? (5d-1) Y

Patent Filed in Foreign Countries? (5d-2) N

Was the assignment forwarded to the contracting officer? (5e) N

Foreign Countries of application (5g-2): none

5a: Hong Zong

5f-1a: Univ of Michigan

5f-c: 1150 W. Medical Ctr Dr

Ann Arbor MI 48109

5a: James R. Baker Jr

5f-1a: Univ of Michigan

5f-c: 1150 West Medical Ctr Dr

Ann Arbor MI 48109

5a: Sascha N. Goonewardena

5f-1a: Univ. of Michigan

5f-c: 1150 W Medical Ctr Dr

Ann Arbor MI 48109

5 UM 5132 – Dendrimer Conjugate and Methotrexate Analogues

Patent Filed in US? (5d-1) Y

Patent Filed in Foreign Countries? (5d-2) N

Was the assignment forwarded to the contracting officer? (5e) N

Foreign Countries of application (5g-2): not determined

5a: Thommey P Thomas

5f-1a: Univ of Michigan

5f-c: 1150 W Medical Ctr Drive

Ann Arbor MI 48109

5a: James R. Baker Jr

5f-1a: Univ of Michigan

5f-c: 1150 W Medical Ctr Dr

Ann Arbor MI 48109

5a: Baohua Huang

5f-1a: Univ of Michigan

5f-c: 1150 W Medical Ctr Dr

Ann Arbor MI 48109

5 UM 5186 Organophosphate Antidote

Patent Filed in US? (5d-1) Y

Patent Filed in Foreign Countries? (5d-2) N

Was the assignment forwarded to the contracting officer? (5e) N

Foreign Countries of application (5g-2): Not determined at this point in time

5a: Pascale R. Leroueil

5f-1a: Univ of Michigan

5f-c: 1150 W Med Ctr Drive

Ann Arbor MI 48109

5a: Abraham F. L. Van Der Spek

5f-1a: Univ of Michigan

5f-c: 1150 W Med Ctr Dr

Ann Arbor MI 48109

5a: James R. Baker Jr

5f-1a: Univ of Michigan

5f-c: 1150 W Medcial Ctr Dr

Ann Arbor MI 48109

5a: Seok Ki Choi

5f-1a: Univ of Michigan

5f-c: 1150 W Medical Ctr Dr

Ann Arbor MI 48109

5 **UM 5217 – Antibacterial Dendrimer**

Patent Filed in US? (5d-1) N

Patent Filed in Foreign Countries? (5d-2) N

Was the assignment forwarded to the contracting officer? (5e) N

Foreign Countries of application (5g-2): none

5a: Seok Ki Choi

5f-1a: Univ of Michigan

5f-c: 1150 W Med Ctr Dr

Ann Arbor MI 48109

5a: James R. Baker Jr

5f-1a: Univ of Michigan

5f-c: 1150 W Med Ctr Dr

Ann Arbor MI 48109

5 **UM 5608 Fluorescent Dye Conjugate**

Patent Filed in US? (5d-1) N

Patent Filed in Foreign Countries? (5d-2) N

Was the assignment forwarded to the contracting officer? (5e) Y

Foreign Countries of application (5g-2):

5a: Hong Zong

5f-1a: Univ of Michigan

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Scientific Progress

See attachment.

Technology Transfer

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Executive Summary:

The overall project consists of two formal phases performed over 5.5 years. Work in Phase I is summarized here. This work focused on developing a battlefield analgesic for soldiers to self-administer. Phase II builds on the results of Phase I and added additional tasks.

In Phase I, which started in March 2007, our efforts were directed towards developing a system to achieve the sustained release of a narcotic (Morphine) and the controlled, feedback release of a narcotic antagonist (Naloxone) in response to hypoxia as described above. Morphine and Naloxone were first modified to form pro-drugs through the attachment of rationally designed chemical modifications. The modifications used for the Morphine pro-drugs were designed to be progressively and consistently cleaved by the esterases present in plasma, while those used in the formation of the Naloxone pro-drugs are cleaved to yield active drug by reduction *only under hypoxic conditions*, therefore serving as the feedback mechanism. Structural variations in the linkers resulted in variations in the rate of the continuous release of Morphine and the hypoxia-activated release of Naloxone. Both sets of pro-drugs were then associated with G5 PAMAM dendrimers to form a delivery platform through either *i*) non-covalent complexation with the polymer or *ii*) covalent conjugation to the dendrimer. The dendrimers in both cases serve to solubilize the drugs and modulate the release kinetics of the pro-drug. A schematic illustrating the family of resulting formulations is displayed in Figure 1. Free pro-drug forms of both Morphine and Naloxone showed faster release kinetics than as dendrimer complexes, which in turn, showed faster release than their conjugate counterparts. These formulations were used in combination to achieve the Phase I milestones of 2.25 mg Morphine/hour and 6 mg Naloxone/hour at pO₂ 18 mmHg.

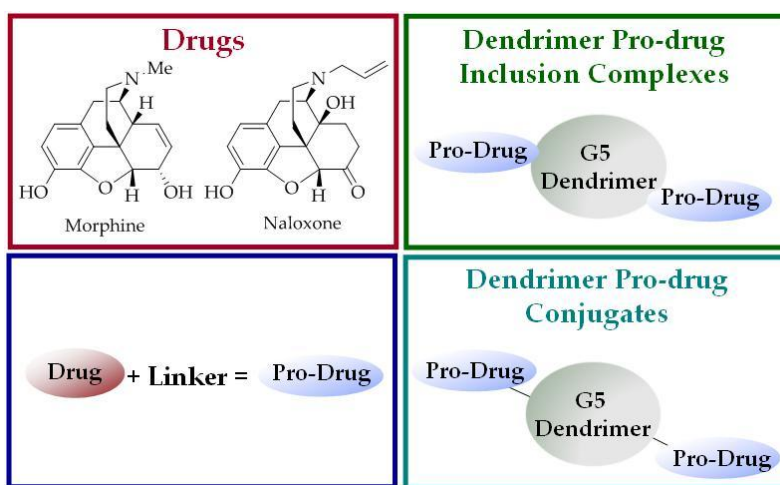


Figure 1. Drug formulations used to achieve desired release kinetics.

Next, we worked on showing proof of concept for the controlled analgesic release system developed previously. First, the synthesis of the Phase I compounds was scaled-up and simplified to enhance yield. Second, *in vitro* studies examining the toxicity and release profiles of these compounds were performed. Third, *in vivo* studies separately examining the efficacy of the Morphine and Naloxone compounds were carried out using animal models. Those compounds that showed promising efficacy were to undergo

ADMET testing. Based on the sum of this information, 1-2 Morphine and Naloxone compounds were selected and carried forward to Phase II.

In Phase II, the research completed within this contract focused on the development of a platform that reverses the effects of an analgesic overdose (Tasks 1-3), and the development a platform that reverses the effects associated with organophosphate poisoning (Task 4).

We successfully scaled up the synthesis of the naloxone prodrug that was designed and tested during Phases I and II, and documented its efficacy in a pig model. Although we had proposed to develop a long-acting form of ketamine, regulatory issues prevented us from moving that project forward. Finally, we designed a novel class of dendrimer-oxime drug conjugates, and evaluated the mechanism by which these conjugates hydrolyze paraoxon.

Key Phase II Accomplishments

Year 1:

- Modified naloxone prodrug synthetic strategy
- Developed *in vivo* pig model for naloxone release studies
- Generated protocol for analyzing naloxone from naloxone prodrug.

Year 2:

- In vivo rescue studies in rats (OSEH subcontract)
- Synthesized large scale batch of naloxone prodrug
- Generated naloxone release data in hypoxic pig model
- Initiated Atropine-Pralidoxime-Extended-Release-Therapeutic (APERT) synthesis

Year 3:

- Concluded naloxone release studies in hypoxic pig model
- Quantified release of naloxone use HPLC MS/MS
- Concluded *in vitro* APERT studies
- Scaled up synthesis of APERT material for planned *in vivo* studies

As a result of this work, we published **15** manuscripts and submitted **12** disclosures and **10** patent applications.

Appendix:

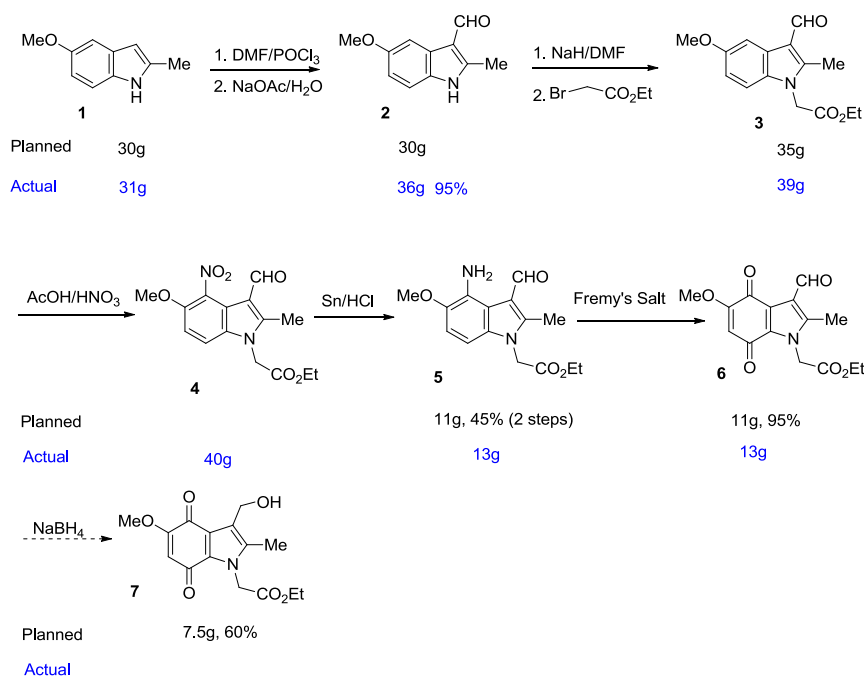
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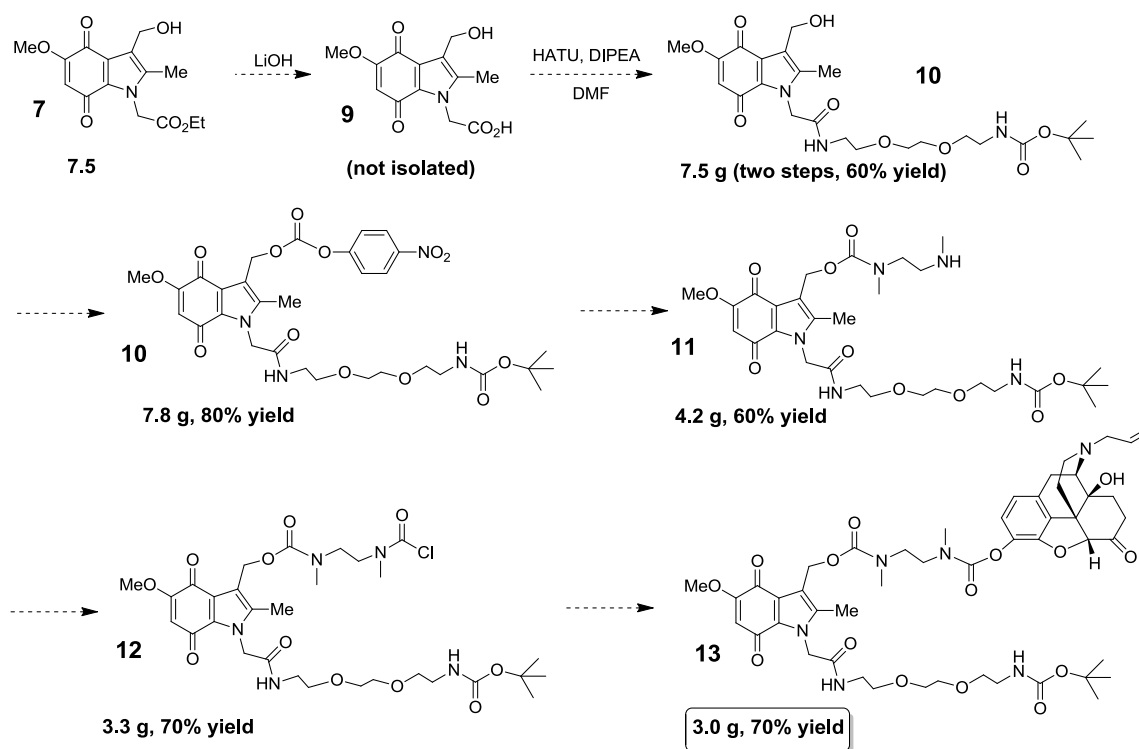
Task 1. Scale-up synthesis of naloxone prodrugs for use in preclinical modeling studies.

Background: The synthesis of indolequinone Naloxone prodrugs has been optimized in phase I and phase II. The scales of the final prodrugs are around 10-20 mg and 900mg, respectively. Specifically, three batches of the dendrimer prodrug conjugates were made, and the scales were 3mg, 10mg, and 15mg. Eight indolequinone Naloxone prodrugs have been synthesized, four of which have a diamine spacer between the drug and the linker containing two carbamate bonds. The other four prodrugs have no such small spacer, and only have one carbonate bond between the drug and the linker. Preliminary release data showed that the carbonate prodrugs are not stable under both hypoxic and normoxic conditions. Based on these results, only prodrugs with diamine spacer were scaled up.

Methods: Three grams of the indolequinone-based prodrug was synthesized using a scale-up procedure developed during Phase II. This procedure consisted of three parts: 1| 9 step-synthesis and the activation of indolequinone linker, 2| activation of the drug, and 3| conjugation of the activated drug with the linker. The final indolequinone-naloxone products were purified by preparation TLC (0.25mm thick, 20x20cm); the conjugates were purified using dialysis & size exclusion chromatography.

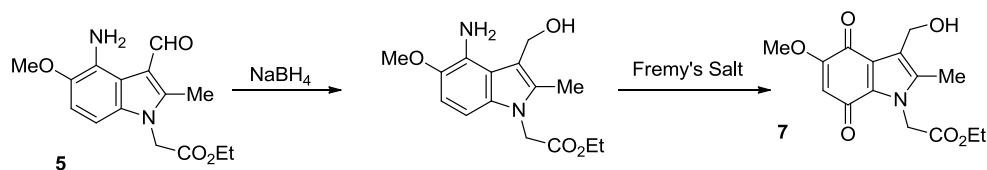


Scheme 1.1| Scale-up synthesis indolequinone linker.



Scheme 1.2 | Scale-up synthesis scheme of naloxone prodrug.

We can only get 20-30% yields at step 6 of synthesis of the indolequinine structure at large scale synthesis. We did some modification trying to increase the yields. The reduction of aldehyde to hydroxyl of step 6 unfortunately has multiple side reactions, and is very sensitive to temperature, reaction time, and the molar equivalent of reducing reagent used. The most probable side reaction is the quinone ring reduction. Because of this, we felt that we could minimize the number of side reactions by performing the reduction reaction first and then convert the aniline to quinone via Fremy's salt. (**Scheme 3**) Using this method, we were able to scale-up the synthesis of compound 7 to 500 mg with a 50-60% yield in two steps.



Scheme 1.3 | Modified synthesis of indolequinone, steps 5 and 6 are reversed.

Results: 2.9 grams of naloxone prodrug has been synthesized by our three chemists. The prodrug was then complexed with dendrimers that were synthesized, purified and characterized in-house. The complex was shown stable in aqueous solution at 4°C for at least 6 months.

Discussion: Given that the dendrimer-naloxone prodrug complex worked sufficiently well in the in vivo model (Task 2), we did not synthesize the dendrimer-naloxne prodrug conjugate. Nonetheless, we do believe that the strategy could still be used to prolong protection against an analgesic overdose.

Conclusion: We have successfully synthesized naloxone prodrug at 3 gram scale, and we can scale-up the synthesis to 10 gram in the future.

Next steps: We will further optimize the synthesis process to increase the yields of some of the synthesis steps.

Task 2: Controlled Release of Naloxone from a Naloxone Prodrug

Background: Respiratory failure is a known and sometimes lethal complication of narcotic overdose. Despite this limitation, narcotics remain the gold standard for treatment of severe acute pain. Untreated severe acute pain has been associated with pathologic central nervous system remodeling, which can lead to the development of chronic pain conditions. Pertinent to battlefield settings, severe acute pain has been associated with long-term psychological effects including post-traumatic stress disorder, as well as depression, anxiety, and substance abuse. Despite these documented consequences of uncontrolled pain, needed medications are often withheld due to safety issues in the field until soldiers or others can be treated in a professionally monitored setting.

Naloxone is an opioid antagonist that is used to treat respiratory depression resulting from a narcotic overdose. Nonetheless, there are limitations associated with its use. For example, the half-life of naloxone is significantly shorter than the half-life of morphine, meaning that more than one naloxone administration would be required to treat a morphine overdose. Thus, the availability and timely administration of naloxone is paramount.

One strategy for easing these availability and administration concerns is to use a naloxone prodrug. Our group previously published the synthesis, characterization and *in vitro* activity of a naloxone prodrug that releases naloxone under hypoxic conditions. In addition, our colleagues have demonstrated the *in vivo* functionality of the prodrug in rats given a lethal dose of morphine. The purpose of the present study was to validate hypoxic release of naloxone in a large animal model where both respiratory and hemodynamic monitoring capabilities exist

Methods: Animals were anesthetized and maintained on isoflurane throughout the experiments. Set up procedures included a tracheostomy for a suture secured 5.5 mm internal diameter endotracheal intubation as well as cut-down access to the bilateral external jugular veins and the femoral artery where a blood draw line, SVO₂ catheter (Hospira Opticath U425C, Lake Forest, IL) and a 20 gauge arterial line were respectively suture secured. Following tracheostomy and line placement, animal monitoring included full respiratory and hemodynamic monitoring using Powerlab with LabChart Pro software (ADI Instruments, Colorado Springs, CO) augmented with SVO₂ monitoring using Q2™ Plus CCO/SO₂ Monitoring (Hospira, Lake Forest, IL).

Three experimental protocols were completed. Our first experimental protocol was designed to assure that naloxone was not releasing prematurely without significant hypoxic challenge. Animals were randomly assigned to groups receiving hypoxic events based on a one minute anoxia time and anoxia maintained until an SVO₂ value of 40 was reached. Animals without hypoxic induction served as controls. Blood sampling was obtained at time 0 followed by the administration of 1mg/kg naloxone prodrug IV at 1 minute. Serial blood sampling was then obtained at 5, 10, 15, 16, 20, 30, 31, 35, 45, 46, 50, 60 minutes for the control and 1 minute hypoxia animals. The hypoxic exposures were 1 minute in duration at times 15, 30, and 45 minutes and blood draws occurred following this minute of hypoxia. In the SVO₂ 40 animals the blood draw following hypoxia was 1 minute after the physiologic hypoxic endpoint (SVO₂ = 40) was achieved. This endpoint was achieved at a mean time of 217 seconds (SEM 3.58 seconds).

In our second experimental protocol, animals were treated the same as protocol #1 above up to the 15 minute mark. At this point, a sustained hypoxic event beginning at 15 minutes was induced in the experimental group with serial blood draws occurred to 30 minutes. Control animals received the same treatment without hypoxia.

In our third experimental protocol animals were treated similar to protocol #2 except that instead of hypoxia, animals were paralyzed and ventilated with the experimental group receiving a non-hypoxic, hypercarbic CO₂ challenge at 15 minutes with serial draws as described above as well as the addition of pH, lactate, and serum CO₂ levels at 0, 20, 25, and 30 minutes.

Results: The animals effectively tolerated the protocols allowing the capture of anticipated data. We demonstrated a fine level of control over animal oxygenation confirmed by respiratory parameters, and oxygenation based on oxygen saturation, and SVO₂ (**Figure 1**)

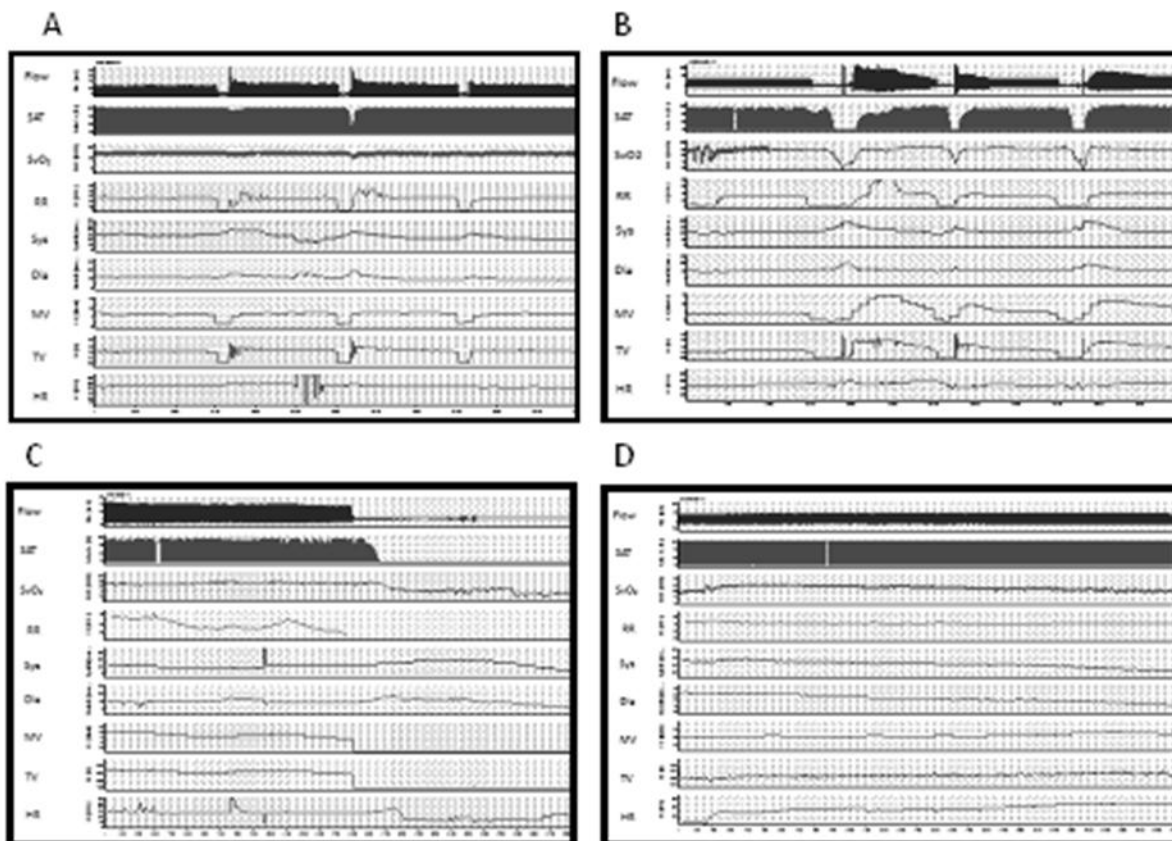


Figure 2.1 | Powerlab tracing of respiratory and hemodynamic parameters with lines as follows: Flow, Oxygen Saturation, SVO₂, Respiratory Rate, Systolic Blood Pressure, Diastolic Blood Pressure, Minute Ventilation, Tidal Volume, Heart Rate. (A) 1 minute Anoxia, (B) Anoxia defined by SVO₂ of 40%, (C) Sustained anoxia, (D) Control

There was greater variability in regards to the degree of hypoxia achieved in the 1 minute hypoxia group compared to the SVO₂ of 40% group where hypoxia was a reliable and repeatable experimental outcome.

In the SVO_2 40 group the hypoxic endpoint was reached at a mean time of 217 seconds (SEM 3.58 seconds). LCMS/MS analysis of the plasma collected at all time points showed that naloxone prodrug and minimal amounts of naloxone were present in the serum. In protocol #1 this data demonstrated no statistically significant increase in naloxone levels in any group when compared to controls confirming that naloxone release was not effected by 1 minute of hypoxia nor hypoxic dives reaching an SVO_2 of 40. The SVO_2 40 group did demonstrate a shouldering effect following hypoxia especially in the 1st and 2nd events but this was not statistically significant and was not above the levels of naloxone noted in the control group (**Figure 2|**)

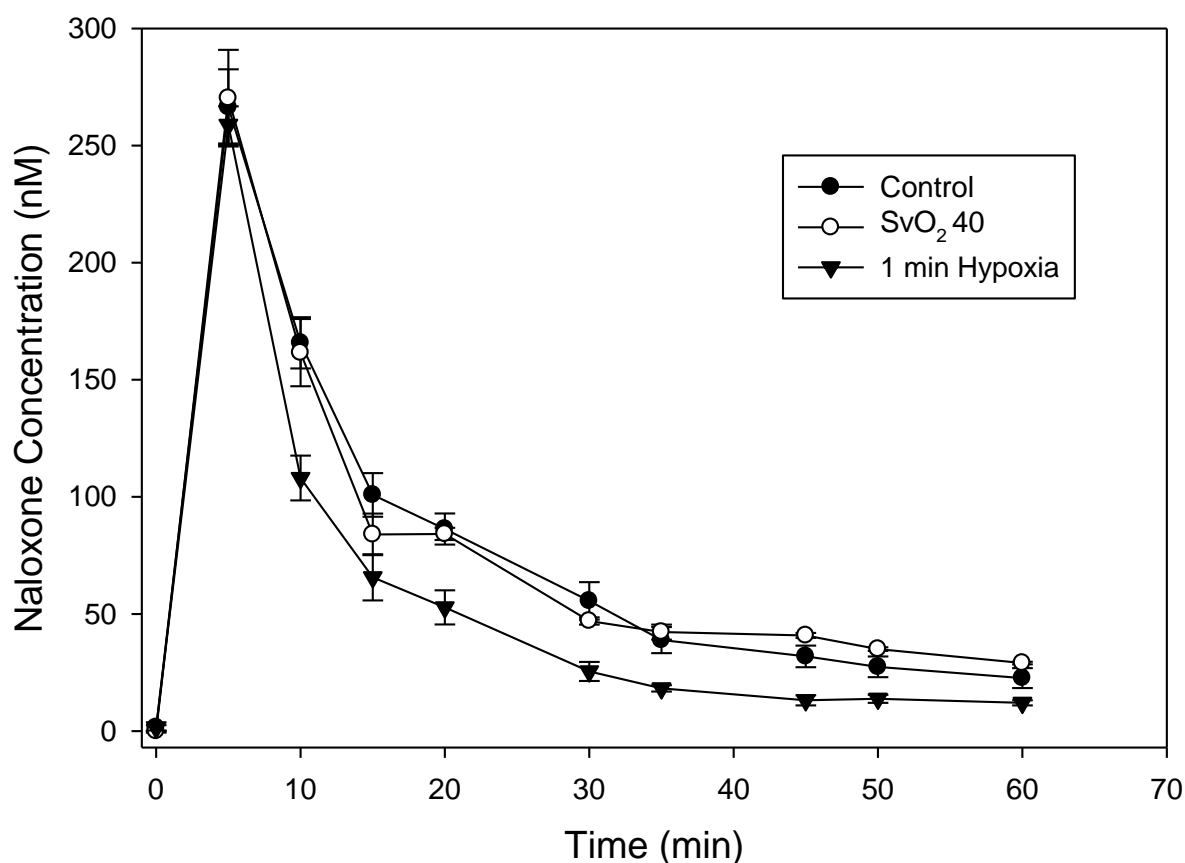


Figure 2.2| Naloxone release following 1 minute and $\text{SvO}_2=40$ hypoxic challenges compared to control.

In protocol #2, we again confirmed prodrug and free naloxone at the 5 minute mark followed by expected redistribution and decreasing serum levels at 10 and 15 minutes similar to protocol #1. In the controls, continued decreases in both components were seen throughout the 30 min experiment. In contrast, animals exposed to sustained hypoxia demonstrated a statistically significant increases in serum concentrations of free naloxone denoting its release and availability under sustained hypoxic challenge (**Figure 2.3|**)

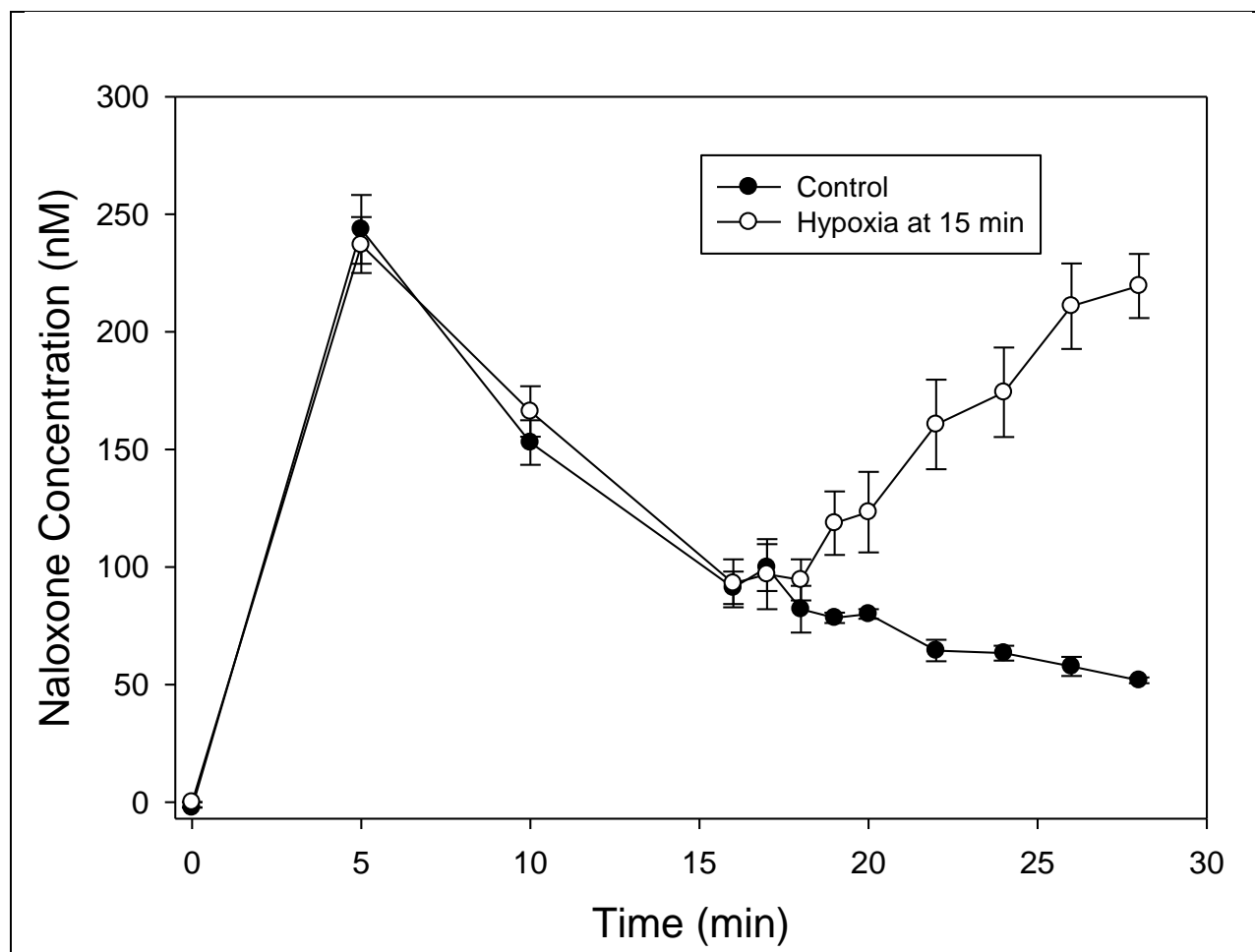


Figure 2.3| In vivo release of naloxone from naloxone prodrug following induction of hypoxia at 15 minutes (clear circles); control animals (black circles) were also given naloxone prodrug but there was no induction of hypoxia. Blood was drawn at regular intervals and serum levels of naloxone were measured using mass spectrometry.

In protocol #3, we again confirmed prodrug and free naloxone at the 5 minute mark followed by expected redistribution and decreasing serum levels at 10 and 15 minutes similar to protocols #1 and #2. In the controls, continued decreases in both components were seen throughout the 30 min experiment. Animals exposed to hypercarbic challenge developed severe hypercarbia and a severe decrease in pH (6.5), with increases in lactate. Naloxone release from prodrug was again noted but at approximately ½ of the levels for hypoxic challenge (**Figure 2.4|**)

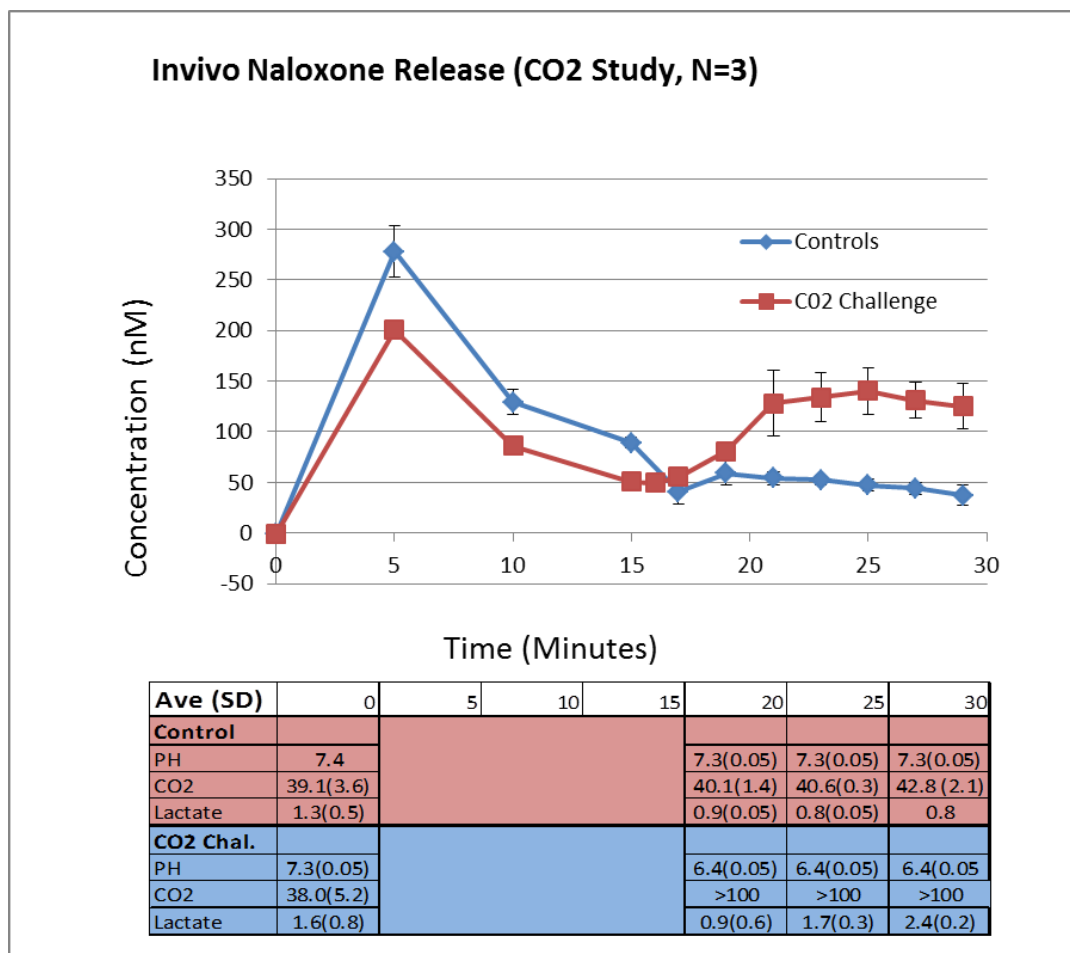


Figure 2.4 In vivo release of naloxone from naloxone prodrug following induction of hypercarbia at 15 minutes (red square); control animals (blue diamond) were also given naloxone prodrug but there was no induction of hypercarbia. Blood was drawn at regular intervals and serum levels of naloxone were measured using mass spectrometry. pH, CO₂, and Lactate levels are also reported.

Discussion: We sought to validate hypoxic release of naloxone from a naloxone prodrug in a controlled hypoxia animal model experiment. Compared to prior in vivo experiments with this prodrug, the current model utilized respiratory and invasive hemodynamic parameters to enhance our monitoring of animal conditions and confirm hypoxia via oxygen saturation and SVO₂ as the trigger for drug release. The data generated confirmed the robust nature of this model and our ability to alter variables and follow animal response in great detail. Additional variations of our experimental protocols included animals maintained on room air and animals maintained on mechanical ventilation with paralysis with hypercarbic challenge leading to a large number of clinical simulations possible in this approach. The model created will allow for the simulation of many clinical scenarios where the effects of hypoxia might be studied.

We further validated the successful creation of a naloxone prodrug where free drug is made increasingly available under hypoxic conditions. A large number of clinical scenarios exist where increased delivery of a drug to hypoxic tissues would be advantageous including myocardial infarction, limb ischemia, stroke, reconstructive flap surgery, etc. The ability to create prodrugs which release in this fashion could have

broad application in these instances particularly when one considers that local tissue concentration of the free drug available due to hypoxia in these tissues would likely lead to a dramatic increase in concentration at the local level. The hypoxic release demonstrated may thereby offer a targeting effect to drug therapy designed to combat local hypoxic injury as well as systemic hypoxia such as that which results in narcotic overdose.

The increased half-life of the naloxone prodrug coupled with the hypoxic release of naloxone may have clinical impact far beyond the scope for which it was originally designed, as an acute narcotic overdose antidote. Agonist/antagonist narcotics have been successfully utilized in clinical settings and the addition of a naloxone prodrug to any pharmacologic indication for narcotics could have the possibility of increasing its safety profile. Given the overwhelming dependence on narcotics in the acute pain setting this need is clearly present.

Defining the hypoxic trigger is certainly one of the challenges that must be considered when developing a drug for hypoxic release particularly when one considers the variable oxygen concentrations in different tissues of the body. In developing our naloxone prodrug we targeted a PO_2 of 15mmHG equating to a O_2 saturation of 21% recognizing that only at critical hypoxemia would drug release be desirable. Our data supported the hypothesis that the prodrug created was only responsive to severe hypoxia since a 1 minute anoxic event and an $SVO_2=40$ event (average 2.5 minutes) of hypoxia had no effect on drug release. Naloxone release reached statistically significant levels 9 minutes after hypoxic induction which is not surprising given the $N=3$ animals per group and a $p < 0.05$ significance level. The clear trends in naloxone increase noted at 4 minutes after hypoxia would be clinically relevant in salvaging narcotic overdose. We believe this data supports a trigger in the desired range for this project's purposes. Other hypoxia targets are certainly feasible and one of the advantages of the model is that we can utilize SVO_2 to specifically tailor our degree of hypoxia. Our animal model therefore is amenable to the documenting and testing of variable degrees of hypoxic challenge which will help delineate the achievement of a specific hypoxia targeted release for any developed drug, regardless of the desired trigger.

Release of naloxone was additionally confirmed in a hypercarbic challenge with resulting acidosis but normal levels of oxygen. At present it is unclear if the release mechanism in this experiment is due to excess CO_2 or acidosis which we plan to define as our next step.

Conclusion: We successfully demonstrated the hypoxic release of naloxone from a naloxone prodrug which was only present during severe hypoxia as compared to lower levels of hypoxia. Drug release has also been documented in hypercarbic acidosis though the individual contribution of CO_2 vs. pH remains to be investigated.

Next steps: We will induce acidosis in a pig model with normal oxygen and CO_2 levels to further define to contributions of acidosis, hypercarbia, and hypoxia on our confirmed naloxone release from prodrug.

Task 2a. *In vivo* rescue studies in rats (*work completed in conjunction with subcontractor George Olsen (OSEH)*).

We used the rat animal model to study the *in vivo* respiratory effects of the newly synthesized naloxone prodrug that was shown to release free naloxone under hypoxic conditions *in vitro* (Huang et al., 2009). Experiments using two chamber, head-out plethysmography in awake animals (Nettleton et al., 2007; Wallisch et al., 2010) were designed to test the hypothesis that the naloxone prodrug releases enough free naloxone *in vivo* under hypoxic conditions caused by a morphine overdose to counteract the severe respiratory depressive effects of morphine without abolishing the analgesic effects. We show that the life-threatening respiratory depression caused by a morphine overdose is counteracted by the co-administration of the naloxone prodrug and attenuated to a level comparable to an analgesically effective and safe dose of morphine. This data suggests that the naloxone prodrug can successfully offset detrimental respiratory side effects of morphine and at the same time retain a safe analgesia levels in the animal.

Methods***Animals and Treatment Groups***

Animal experiments were approved by the Oregon Health & Science University's Institutional Animal Care and Use Committee (OHSU IACUC) and the United States Army Medical Research and Materiel Command's Animal Care and Use Review Office (USAMRMC ACURO). Experiments were conducted in accordance with National Institutes of Health guidelines. Male Sprague-Dawley rats (11 weeks, 325-350 g) were purchased from Charles River Laboratories (Wilmington, MA) and housed in pairs with 12 hr controlled light/dark cycles and *ad libitum* food and water at Oregon Health & Science University for at least 48 h prior to the experiments. Animals were randomly assigned to the treatment groups for respiratory, pharmacokinetic and rescue experiments. Respiratory testing consisted of six treatment groups in which animals received a single i.v. injection of 1 ml/kg of a saline solution (SAL, 0.9%), naloxone (NLX; 1 mg/kg), naloxone prodrug (NPD; 1mg/kg naloxone content), morphine (MOR 4; 4 mg/kg), morphine overdose with naloxone (MOR 40 + NLX; 40 mg/kg morphine, 1mg/kg naloxone), or morphine overdose with naloxone prodrug (MOR 40 + NPD; 40 mg/kg morphine, 1mg/kg naloxone content) into the lateral tail vein. Pharmacokinetic analysis was done for the morphine overdose test groups, MOR 40 + NLX and MOR 40 + NPD. In addition for the rescue studies animals received an i.v. injection of a morphine overdose (MOR 40; 40 mg/kg). The animals' breathing was constantly monitored. This injection was followed by an i.v. rescue injection of NLX or NPD (1 mg/kg naloxone content) at the indicated times of rescue. Two respiratory criteria were used to determine the time of rescue: 1) inspiratory minute ventilation fell below 50% of room air baseline breathing for 1 minute, or 2) no increase in inspiratory minute ventilation (CO₂ response) was seen in two consecutive CO₂ challenges.

Respiratory Measurements

Animals were placed in a non-invasive, head-out, two chamber plethysmograph (Buxco Electronics, Sharon, CT). Importantly, the head chamber allowed alternating administration of the two study gases. Room air (RA; 21% O₂, balance N₂) was administered at a steady flow rate of 1.8 l/min; during hypercapnic challenge elevated CO₂ (CO₂; 5% CO₂, 30% O₂, balance N₂) was administered at 2.0 l/min.

A pressure transducer continuously recorded animal breathing movements from the separate body chamber and averaged the data every 5 sec. Directly measured parameters were breathing frequency (f_R ; breaths·min⁻¹), inspiratory time (T_I ; sec), expiratory time (T_E ; sec), and air flow (ml/sec), which were used to calculate tidal volume (V_T ; ml), inspiratory minute ventilation (V_I ; ml·min⁻¹), and inspiratory effort (V_T/T_I ; ml·sec⁻¹) using FinePointe software version 1.6.2 (Buxco Electronics, Sharon, CT). Briefly, rats were placed in the plethysmograph for 15 min acclimation period at RA, followed by a 10 min RA and 5 min CO₂ baseline measurement period. Immediately after CO₂ baseline recording, the back of the body chamber was opened to access the tail vein of the animal for the injection of the test substance, while the animal remained in the plethysmograph. After the injection, alternating measurement periods of 10 min RA and 5 min CO₂ were continued for 2 hours, followed by a final 5 min RA period before the animal was taken out of the plethysmograph. Data reported is the average of the last 3 minutes of each measurement period.

Pharmacokinetic Analysis

Rats were injected i.v. into either of the two lateral tail veins with MOR 40 + NLX or MOR 40 + NPD. Blood volumes of 100-150 μ l were drawn through puncturing either the lateral- or the medial-saphenous vein at indicated time points after injections. Each vein was only used to draw 1 or 2 blood samples. Blood was collected in heparinized capillary tubes with separation gel (Ram Scientific, Inc, Yonkers, NY) and centrifuged for 2 min at 12,000g. Plasma fraction on top of the gel was removed and two 15 μ l samples were collected and added to 35 μ l of methanol that contained the internal standard, d₃-morphine. Tubes were vortexed and placed on ice for 10 minutes. The samples were centrifuged at 12,000g for 15 min and the supernatant was collected in autosampler vials for LC-MS/MS analysis.

The LC-MS/MS system consisted of an Accela HPLC interfaced to a TSQ Quantum Discovery (ThermoFisher, Waltham, MA) mass spectrometer. The analytical column was a Synergi HydroRP C18 80 Å (4 μ , 150 x 3 mm, Phenomenex, Torrance, CA). The mobile phase consisted of 0.02% formic acid (solvent A) and 0.02% formic acid in acetonitrile (solvent B). Initial conditions were 3% solvent B for 2 min that was increased to 95% solvent B over 6 min and held for 2 min. The system was returned to 3% solvent B and equilibrated for 4 min. The flow rate was 0.30 ml/min. The mass spectrometer was equipped with an electrospray interface (ESI) and operated in the positive ionization mode. The source parameters were: spray voltage 3500; sheath and aux gas flow rates, 30 and 5, respectively; tube lens offset, 174 V; and capillary temperature, 275°C. This system was set up in selected reaction monitoring (SRM) mode, monitoring the transitions m/z 286.1→m/z 165.0 and m/z 286.1→m/z 201.0 for morphine, m/z 328.0→m/z 253.0 for naloxone, and m/z 289.1→m/z 165.0 and m/z 289.1→m/z 201.0, for the internal standard d₃-morphine. Scan event settings were scan width 1.5 m/z, scan time 0.5s, CID gas pressure of 1.0, collision energy of 35 and Q1/Q3 peak width of 0.7. Calibration curves of the peak area ratios of analyte/internal standard versus analyte concentration were generated in naïve plasma for each set of samples. Regression coefficients of ≥ 0.998 were obtained and the relative standard deviation for the lowest calibration curve point (10 ng/ml for naloxone and 50 ng/ml for morphine) was less than 20%. Data acquisition and analysis were performed using the Xcalibur software version 2.0 (ThermoFisher, Waltham, MA).

Data Analysis and Statistics

Respiratory data was collected continuously during both the 10 min RA and 5 min CO₂ recording periods and averaged every 5 sec. To ensure steady-state had been reached, the reported and analyzed data are the averages of the last 3 min of each recording period (i.e. for RA min 8-10, 23-25, 38-40, etc. and for CO₂ min 13-15, 28-30, 43-45, etc.). For statistical analysis and comparison among treatment groups, data for the post injection RA and CO₂ time course was normalized to the average RA baseline for each treatment group. All values are reported as the mean \pm standard error. Statistical significant differences for all respiratory parameters were determined using two-way repeated measure analysis of variance (RM ANOVA) with factors of treatment and time. Post-hoc analysis for statistical difference ($p < 0.05$) was performed using the Holm-Sidak method using either SAL or MOR 4 as control groups (Sigma Stat 3.11, SysStat Software Inc., Richmond, CA). For post-hoc analysis, all treatment groups were compared to SAL as control for treatment and treatment-time interaction, in addition all morphine treatment groups were compared to MOR 4 as a control again for treatment and treatment-time interaction. For the rescue studies, two-way ANOVA with factors of treatment and time was used to analyze respiratory parameters before and after the rescue injection.

Pharmacokinetic data analysis was performed using PK Solver 2.0 for MS Excel (Zhang et al., 2010), followed by the Student's *t*-test with treatment as factor using Sigma Stat 3.1 (Systat Software Inc, San Jose, CA)

Results

Animal weight, age and treatment

Male Sprague-Dawley rats were 79-86 days old at the day of the experiment and weighed between 346-425 g (383 \pm 2 g). Neither age nor weight of the animals was different among the treatment groups. The average weight for each treatment group is shown in **Table 2a.1**.

Treatment doses were determined in preliminary studies or are based on available literature. Preliminary analgesia studies in our laboratory and by others (Morgan et al., 2006) have shown that a single i.v. injection of 4 mg/kg of morphine (MOR 4) provides significant analgesia for a period of 1.5 to 2 hrs post injection. A ten times overdose of 40 mg/kg of morphine was chosen and approved by the OHSU IACUC, because this dose can cause life-threatening respiratory depression, while it is still below the

Treatment	N	Weight \pm SEM (g)
<i>Respiratory Study Groups</i>		
SAL	4	381 \pm 13
NLX	4	390 \pm 3
NPD	6	390 \pm 5
MOR 4	5	372 \pm 8
MOR 40 + NLX	6	383 \pm 5
MOR 40 + NPD	6	386 \pm 10
<i>Pharmacokinetic Study Groups</i>		
MOR 40 + NLX	5	378 \pm 6
MOR 40 + NPD	6	387 \pm 8
<i>Overdose Rescue Study Groups</i>		
MOR 40 \rightarrow NLX	6	382 \pm 5
MOR 40 \rightarrow NPD	6	384 \pm 9
Total	54	383 \pm 2

Table 2a.1: Animal weight for all treatment groups within the respiratory, pharmacokinetic and overdose rescue studies. (SAL: Saline; NLX: Naloxone 1 mg/kg; NPD: Naloxone Prodrug 1 mg/kg; MOR 4: Morphine 4 mg/kg; MOR 40: Morphine 40 mg/kg)

reported LD₅₀ of 64-140 mg/kg for i.v. morphine in rats (Jackson 1952; Borron et al., 2002; Strandberg et al., 2006). The dose of 1 mg/kg of naloxone chosen is slightly higher than the up to 0.6 mg/kg used to antagonize morphine overdoses of 40 mg/kg (van den Hoogen and Colpaert 1986) make sure that the respiratory depression of the morphine dose is almost completely reversed and to account for the fact that the total naloxone content of the NPD is not immediately available (Huang et al., 2009).

Respiratory Measurements

Baseline Measurement

Baseline respiratory measurements were taken directly prior to the injection of the test substance and after the animal became acclimated to the plethysmograph chamber. **Table 2** shows the RA and CO₂ baseline values for inspiratory minute volume V_I, frequency f_R, tidal Volume V_T, inspiratory effort IE, and inspiratory and expiratory time, T_I and T_E. There were no statistical differences among the different treatment groups.

Treatment	N	Minute Ventilation		Frequency		Tidal Volume		Inspiratory Time		Expiratory Time		Inspiratory Effort	
		[ml/min]		(breath/min)		(ml)		(sec)		(sec)		(ml/sec)	
Respiratory Study Groups													
		RA	CO ₂	RA	CO ₂	RA	CO ₂	RA	CO ₂	RA	CO ₂	RA	CO ₂
SAL	4	285±17	695±91	104±6	166±9	2.8±0.2	4.3±0.4	0.26±0.01	0.20±0.01	0.33±0.03	0.18±0.01	10.9±1.5	22.3±3.0
NLX	4	295±18	723±45	122±3	184±15	2.5±0.2	4.0±0.1	0.21±0.01	0.17±0.01	0.30±0.03	0.17±0.01	11.8±1.5	24.3±1.6
NPD	6	300±18	627±102	119±7	165±20	2.4±0.1	3.8±0.2	0.24±0.03	0.21±0.03	0.29±0.02	0.20±0.02	11.2±1.1	20.6±3.5
MOR 4	5	302±21	706±65	125±4	190±9	2.4±0.1	3.8±0.4	0.20±0.01	0.17±0.01	0.29±0.02	0.16±0.01	12.3±0.9	22.7±1.6
MOR 40 + NLX	6	322±20	698±73	120±7	178±14	2.7±0.1	4.0±0.3	0.23±0.02	0.18±0.02	0.29±0.02	0.18±0.02	12.5±1.2	23.4±2.6
MOR 40 + NPD	6	277±21	573±51	123±8	189±10	2.4±0.2	3.1±0.2	0.21±0.01	0.16±0.01	0.33±0.03	0.17±0.01	12.0±1.3	19.8±1.9
Overdose Rescue Study Groups													
MOR 40→NLX	6	319±17	552±37	131±8	163±10	2.5±0.1	3.4±0.1	0.21±0.01	0.19±0.01	0.27±0.02	0.19±0.01	12.0±0.5	18.1±1.3
MOR 40→NPD	6	281±14	572±47	112±4	155±8	2.6±0.1	3.8±0.2	0.24±0.02	0.20±0.01	0.32±0.01	0.20±0.01	11.2±1.1	19.1±1.8

Table 2a.2: Respiratory baseline data for room air breathing (RA) and 5% CO₂ challenge (CO₂) for each treatment group. There were no significant differences. (SAL: Saline; NLX: Naloxone 1 mg/kg; NPD: Naloxone Prodrug 1 mg/kg; MOR 4: Morphine 4 mg/kg; MOR 40: Morphine 40 mg/kg)

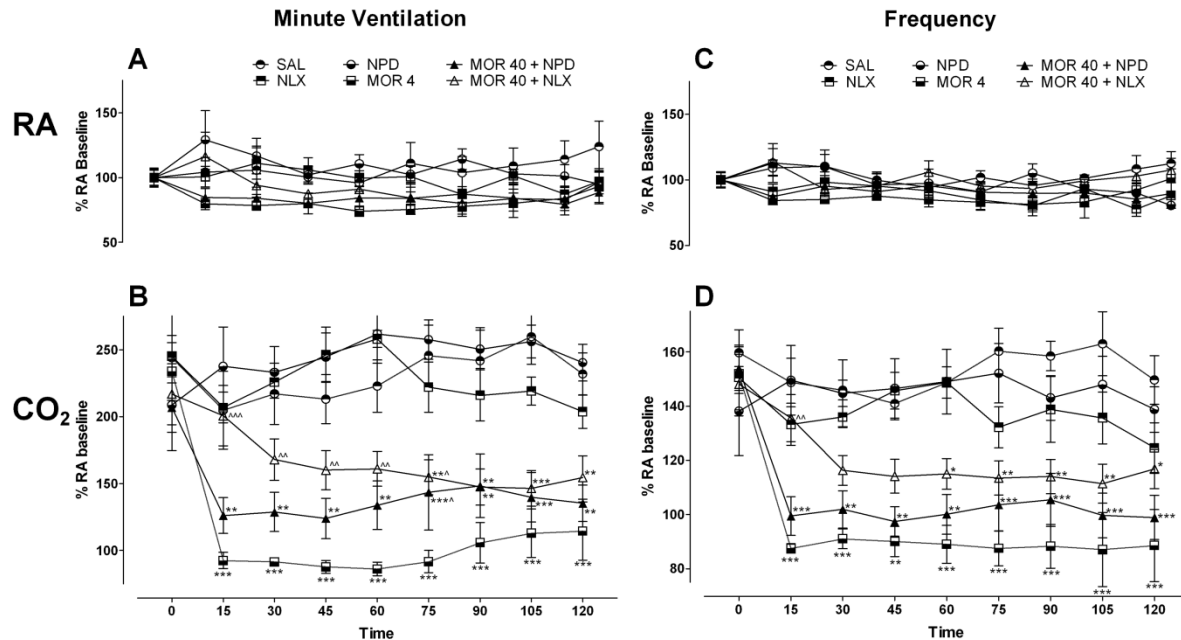
Inspiratory Minute Ventilation and Frequency

Figure 2a.1. Inspiratory minute volume (V_I , left panel) and frequency (f_R , right panel) time-action curves for RA breathing (top) and 5% CO_2 challenge (bottom). Data is normalized to RA baseline for all parameters. No differences are seen among all groups for RA breathing for both V_I (A) and f_R (C). During CO_2 challenge both MOR 4 and MOR 40 + NPD treatments cause significantly decreased V_I (C) and f_R (D) responses compared to SAL during the complete post injection time course, while MOR 40 + NLX treatment only causes a decreased response in the second hour of the time course. In addition, the first hour response of MOR 40 + NLX is significantly different from MOR 4 response. Both negative controls NPD and NLX were not different from SAL. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs SAL; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs MO

For V_I there were main effects for treatment and time, and a treatment time interaction ($p < 0.001$ for all comparisons) during 5% CO_2 challenge. No differences between treatments were seen for V_I during RA breathing (**Figure 2a.1A**). All three morphine groups were significantly different from the SAL control during CO_2 challenge ($p < 0.001$ for MOR 4 and MOR 40 + NPD, $p < 0.01$ for MOR 40 + NLX) (**Figure 2a.1B**). Animals treated with MOR 4 and MOR 40 + NPD had significantly decreased V_I response to the CO_2 challenge compared to SAL for the whole two hour recording period, while MOR 40 + NLX treated animals were only different from SAL during the last 45 minutes of testing. Comparison of the MOR treatment groups showed that only MOR 40 + NLX treatment was different from MOR 4 ($p < 0.05$) with animals treated with MOR 40 + NLX having a better V_I response to the 5% CO_2 challenge for the first 75 min post injection. MOR 4 and MOR 40 + NPD groups were not different from each other.

Similar to V_I there were main effects for f_R for treatment and time, and a treatment time interaction ($p < 0.001$ for all comparisons) during 5% CO_2 challenge (**Figure 2a.1D**), but no differences during RA breathing (**Figure 2a.1C**). All morphine treatment groups had a decreased f_R response compared to SAL ($p < 0.001$ for MOR 4 and MOR 40 + NPD, $p < 0.01$ for MOR 40 + NLX). MOR 40 + NLX treated

animals showed a decreased f_R response to CO_2 challenge in the second hour (starting at 60 min) post injection, while the MOR 4 and MOR 40 + NPD groups showed this decrease for the whole two hour period. The statistical comparison of f_R response for the morphine groups shows no main effect for treatment among the groups, but a treatment time interaction for the comparison of MOR 4 and MOR 4 + NLX treated animals in the first 15 min of the time course.

Both analyses for V_I and f_R show that the morphine control animals (MOR 4) and the morphine overdose with naloxone prodrug rescued animals (MOR 40 + NPD) have decreased V_I and f_R responses to the CO_2 challenge compared to SAL control animals for the duration of the whole time course, while they are not different from each other. The morphine overdose animals simultaneously injected with naloxone (MOR 40 + NLX) instead of the naloxone prodrug show a complete reversal of the morphine effects in the first 45 (f_R) to 60 min (V_I) after injection.

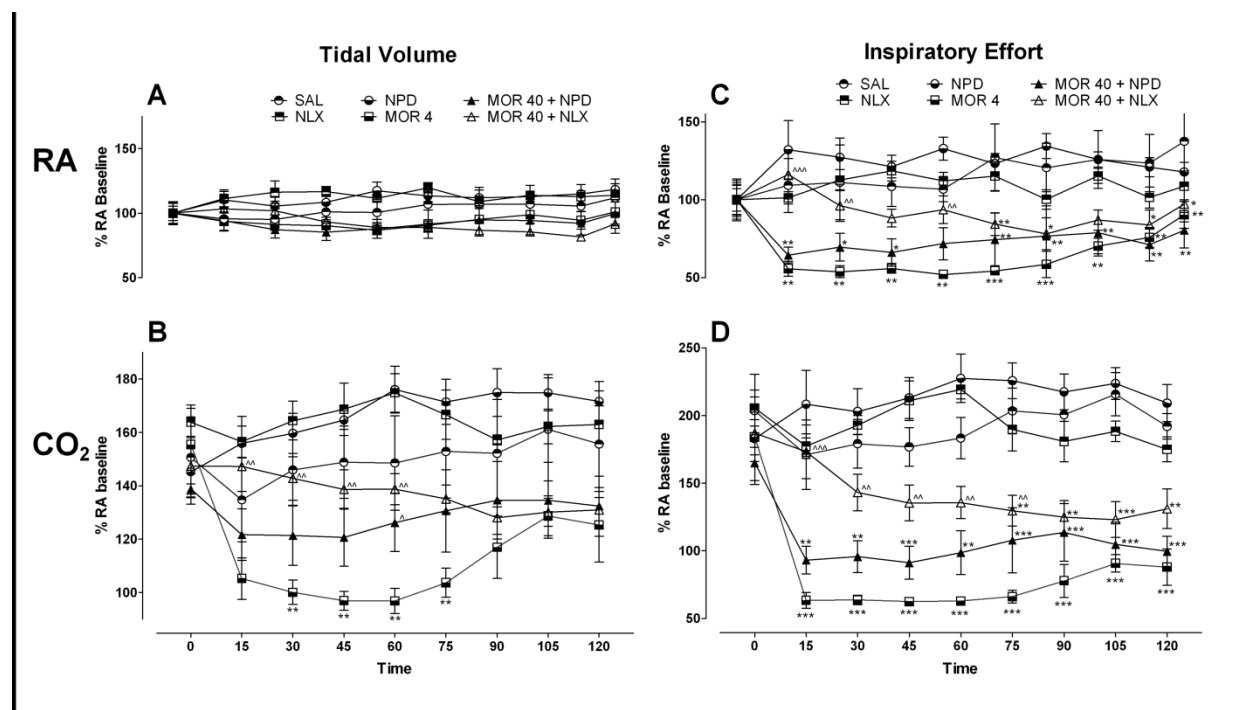


Figure 2a.2. Tidal Volume (V_T , left panel) and inspiratory effort (V_T/T_I , right panel) time-action curves for RA breathing (top) and 5% CO_2 challenge (bottom). Data is normalized to RA baseline for all parameters. No differences are seen among all groups for RA breathing for V_T (A). During CO_2 challenge (B) MOR 4 treatment causes a significantly reduced response for the mid-section of the time course compared to SAL that is also different from the MOR 40 + NLX treatment. Effects on inspiratory effort (IE) are seen for both RA breathing (C) and 5% CO_2 challenge (D) with MOR 4 and MOR 40 + NPD treatment causing significantly decreased IE responses compared to SAL for the two hour time course, while MOR 40 + NLX treatment only causes a decreased response in the second hour. In addition, effects on IE during both breathing challenges are significantly less for the MOR 40 + NLX treatment group compared to MOR 4 for the first hour. Both negative controls NPD and NLX were not different from SAL. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs SAL; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs MOR

Tidal Volume and Inspiratory Effort

During RA breathing, V_T was not different among all treatment groups (**Figure 2A**). There were main effects for V_T for treatment ($p < 0.01$) and time ($p < 0.001$), and a treatment time interaction ($p < 0.001$) during the 5% CO_2 challenge (**Figure 2a.2B**). While the NPD and NLX control animals tend to have a slightly, but not significantly higher V_T response to CO_2 and the morphine groups tend to have slightly

diminished response compared to SAL control animals, only the MOR 4 treated animals showed a significant decrease in their V_T response to CO_2 compared to SAL during the middle part of the post injection time course (30-75 min). When compared to MOR 4, the MOR 40 + NLX group was statistically different during the first 60 min, while MOR 4 and MOR 40 + NPD were not different from each other with the exception of the 60 min time point.

For inspiratory effort IE, which is calculated as V_T/T_I , there were main effects during both RA breathing and 5% CO_2 challenge (**Figure 2a.2C,D**). There was a main effect of treatment ($p < 0.001$) and a treatment time interaction ($p < 0.001$) for both RA and CO_2 ; in addition there was a main effect of time ($p < 0.001$) for IE during the CO_2 challenge. Compared to SAL control animals, both MOR 4 and MOR 40 + NPD treated animals had a statistically reduced inspiratory effort during RA breathing and CO_2 challenge ($p < 0.001$ for all comparisons, except for $p < 0.01$ for MOR 40 + NPD at RA) with the significant decreases in IE response present for the complete two hour time course. The reduced IE compared to SAL was also seen during the second hour of the time course (starting at 75 min) for the MOR 40 + NLX treatment group showing a treatment time interaction, while the effect of treatment reached an overall statistical difference for the comparison of SAL and MOR 40 + NLX only during CO_2 challenge ($p < 0.05$).

The morphine containing treatment groups MOR 4 and MOR 40 + NPD were not different from each other breathing either study gas. In contrast, the MOR 40 overdosed and NLX co-injected animals were statistically different from MOR 4 during 5% CO_2 challenge ($p < 0.001$). The inspiratory effort of MOR 40 + NLX animals was statistically greater compared to MOR 4 during the first 75 min of the time course when breathing 5% CO_2 . In addition a treatment time interaction was seen during RA breathing for these two treatment groups at RA at individual time points during the first hour post injection, but these differences were not great enough to cause a treatment effect for the comparison of MOR 4 and MOR 40 + NLX ($p = 0.06$).

Statistical analysis of the two parameters V_T and V_T/T_I (IE) show that the MOR 4 control animals and the MOR 40 + NPD treated animals are not different from each other, while they are different from the SAL control during RA breathing and during CO_2 challenge (IE only). In contrast, the MOR 40 + NLX treatment group is different from MOR 4 during the first hour post injection during RA breathing (IE only) and CO_2 challenge (V_T and IE), but not different from SAL. This shows again an almost complete attenuation of the morphine induced respiratory depression for the NLX co-injected group, at least during the first hour. The co-injection of NPD with the morphine overdose only reduces the respiratory effects of the overdose to the level of the effects of a ten times lower dose.

Inspiratory and Expiratory Time

There were main effects for treatment, time and a time treatment interaction for T_I for both RA breathing (**Figure 2a.3A**) and CO_2 challenge (**Figure 2a.3B**) ($p < 0.001$ for all comparisons). Only the MOR 4 and MOR 40 + NPD treatment groups were different from SAL ($p < 0.001$ for all comparisons, except $p < 0.01$ for MOR 40 + NPD at RA) showing a prolonged T_I for both RA and CO_2 . MOR 40 + NLX did not show a significant increase in T_I and was not statistically different from SAL control animals, but was different from MOR 4 control animals during the first 90 min (RA, $p < 0.001$) or the complete two hour

time course (CO_2 , $p < 0.001$). MOR 40 + NPD animals also showed a lower increase in T_I response compared to MOR 4, which reached statistical significance during CO_2 challenge ($p < 0.05$), but not while breathing RA ($p = 0.08$).

For T_E there were main effects of treatment ($p < 0.01$), time ($p < 0.001$), and a time treatment interaction ($p < 0.001$) during RA breathing (**Figure 2a.3C**). All though none of the treatment groups were different from the SAL control animals, NLX and NPD control groups tended to have somewhat longer, MOR containing groups somewhat shorter T_E . During 5% CO_2 challenge, there was a main effect of time and a time treatment interaction ($p < 0.001$ for both comparisons), but no main effect of treatment ($p = 0.07$) (Fig 3D). Only the MOR 4 treated animals had a 30 min period at the beginning of the second hour where T_E was significantly increased compared to SAL control animals.

The statistical analysis of changes in inspiratory time compared to baseline show the only differences in respiratory response between the MOR 4 control group and the MOR 40 overdose with NPD co-injected animals, while both again are different from the NLX co-injection group of animals.

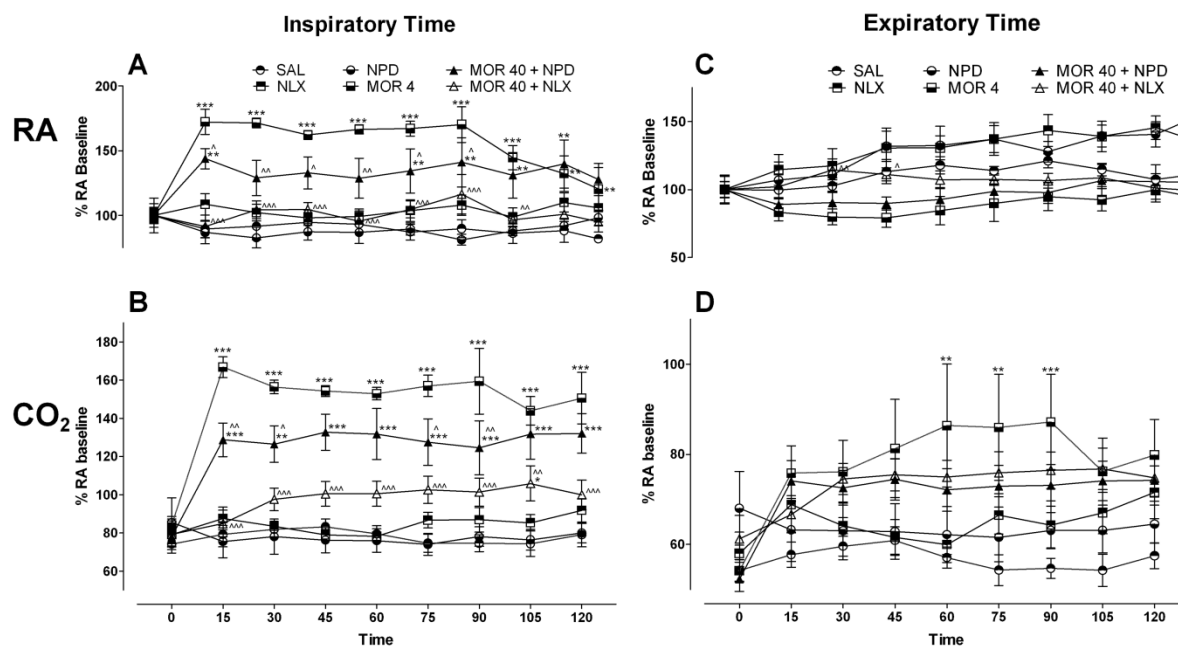


Figure 2a.3. Inspiratory time (T_I , left panel) and expiratory time (T_E , right panel) time-action curves for RA breathing (top) and 5% CO_2 challenge (bottom). Data is normalized to RA baseline for all parameters. T_I increases significantly for RA breathing (A) and CO_2 challenge (B) for both MOR 4 and MOR 40 + NPD compared to SAL, with the magnitude of the increase being bigger for the MOR 4 group than for the MOR 40 + NPD treated animals. T_I is not different from SAL for MOR 40 + NLX treated animals and NPD and NLX controls. For T_E response during RA (C) no differences are seen among treatment groups compared to SAL. During CO_2 challenge (D) an increased T_E is seen in the second hour of the time course for MOR 4 only, while the rest of the treatments is not different from SAL. ** $p < 0.01$, *** $p < 0.001$ vs SAL; # $p < 0.05$, ## $p < 0.01$ vs MOR

Pharmacokinetic Blood Sampling

To compare the concentration of morphine and naloxone in the blood between the MOR 40 overdose groups co-injected with NPD or NLX, blood samples were taken for 3 hours following the i.v. injection and analyzed by LC-MS/MS. For both groups, we analyzed the morphine blood concentrations plus the free naloxone or released naloxone blood concentrations, respectively. The reported concentrations do not include the naloxone prodrug.

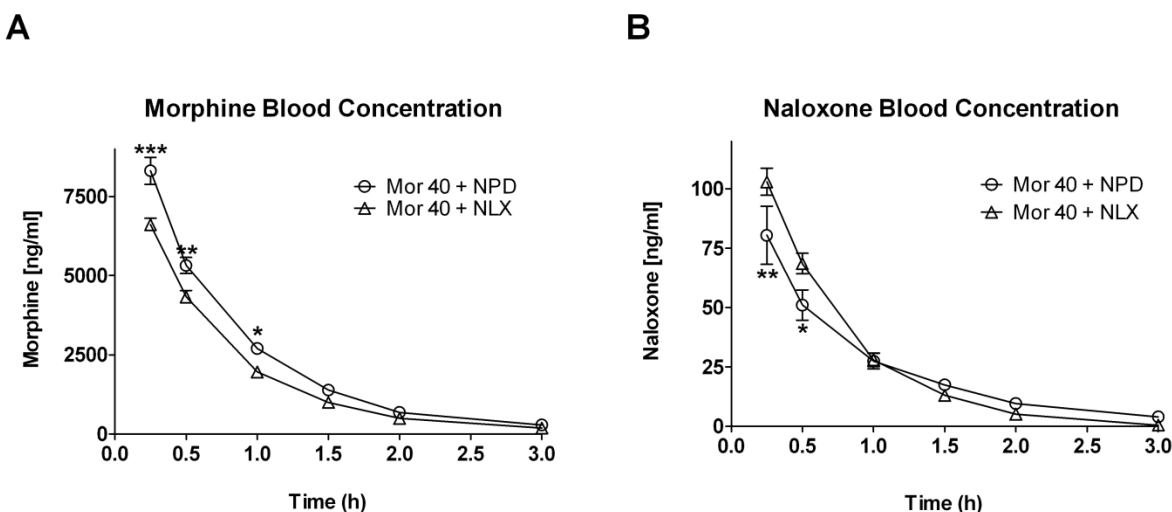


Figure 2a.4. Morphine (A) and naloxone (B) blood concentrations were measured for a 3 hour time course after an i.v. bolus injection of a morphine overdose (40 mg/kg) paired with either free naloxone (open circles) or naloxone prodrug (triangles). Both morphine and naloxone show a first order elimination kinetic (PK solver 2.0 for MS Excel). Morphine blood concentrations are higher in the first hour for the NPD co-injection animals (A). Both NPD and NLX injection contain the same concentrations of naloxone, but during the first 30 min naloxone concentration in NPD injected animals are significantly lower, because of the slow release of naloxone. Effects on pharmacokinetic parameters are shown in **Table 3.**; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs MOR + NLX

Drug / Injection	N	Conc. at t=0 (C_0) (ng/ml)	Elimination Constant (min^{-1})	Half-Life Time ($t_{1/2}$) (min)	Clearance CL ($\text{ml kg}^{-1} \text{min}^{-1}$)	Vol. of Dist. @ Steady State (V_{ss}) (l/kg)
<i>Morphine</i>						
MOR 40 + NLX	5	10160 ± 1080	0.021 ± 0.001	32 ± 2	101 ± 9	4.1 ± 0.4
MOR 40 + NPD	6	12720 ± 2100*	0.020 ± 0.002	35 ± 4	81 ± 13*	3.5 ± 0.6†
<i>Naloxone</i>						
MOR 40 + NLX	5	157 ± 34	0.027 ± 0.004	26 ± 4	174 ± 19	6.1 ± 1.0
MOR 40 + NPD	6	128 ± 59	0.018 ± 0.007*	42 ± 13*	194 ± 50	10.9 ± 5.0†

* $p < 0.05$ vs. MOR 40 + NLX; † $p < 0.1$ vs. MOR 40 + NLX

Table 2a.3: Pharmacokinetic data for the morphine overdose with naloxone or naloxone prodrug co-administration. (NLX: Naloxone 1 mg/kg; NPD: Naloxone Prodrug 1 mg/kg; MOR 40: Morphine 40 mg/kg)

Figure 2a.4 shows the blood concentration of morphine (**Figure 2a.4A**) and naloxone (**Figure 2a.4B**) for animals injected with either MOR 40 + NLX or MOR 40 + NPD. Both concentration profiles are consistent with an i.v. bolus injection and first order elimination kinetic. Morphine concentrations were higher during the first hour post injection for animals co-injected with NPD, while naloxone concentrations were lower in the first 30 min for the same animals. Pharmacokinetic analysis of this data with PK Solver 2.0 for Excel using a non-compartmental analysis of an i.v. bolus injection is shown in **Table 2a.3**. The presence of NPD has no effect on the half-life and the elimination constant of morphine compared to the NLX group, but significantly changes the extrapolated concentration at the time of injection, significantly reduces the clearance and also causes a slightly reduced volume of distribution at steady state. Naloxone released from the prodrug shows a different pharmacokinetic profile compared to free naloxone. This is shown by an increased half-life time and a reduced elimination constant for naloxone in the animals injected with MOR 40 + NPD. In addition, the volume of distribution shows a trend to be increased. Interestingly, but not surprisingly, the variance of the data for the MOR 40 + NPD treated animals is greater for all pharmacokinetic parameters compared to MOR 40 + NLX treated animals, suggesting some variation among animals in the metabolism of the NPD, as well as the occurrence of the first severe hypoxic event that triggers the naloxone release.

Morphine Overdose and Rescue Studies

A second respiratory study was performed where animals were given a morphine overdose of 40 mg/kg first, followed by either a NLX or NPD rescue injection. This morphine dose caused severe or life-threatening respiratory depression as defined by our rescue criteria of a V_I reduced to 50% or less of RA baseline or no apparent V_I response during two consecutive CO_2 challenges in 11 of 12 test animals (6 per group). Time to reach the rescue criteria varied from 4 to 47 min post morphine overdose. Seven animals received a rescue injection after their V_I dropped below 50% (NLX n = 4; NPD n = 3), one animal's V_I dropped below 50% of RA baseline during a CO_2 challenge (NPD). Three animals received a rescue injection after not responding to two CO_2 challenges (NLX n = 1; NPD n = 2). One animal showed strong respiratory effects, but did not meet our criteria for rescue during the two hour time course. This animal was given a NLX injection 10 min before the 2 hour time point. One animal in each group recovered slowly and was given a second injection of naloxone. These injections were given to minimize stress on the animals.

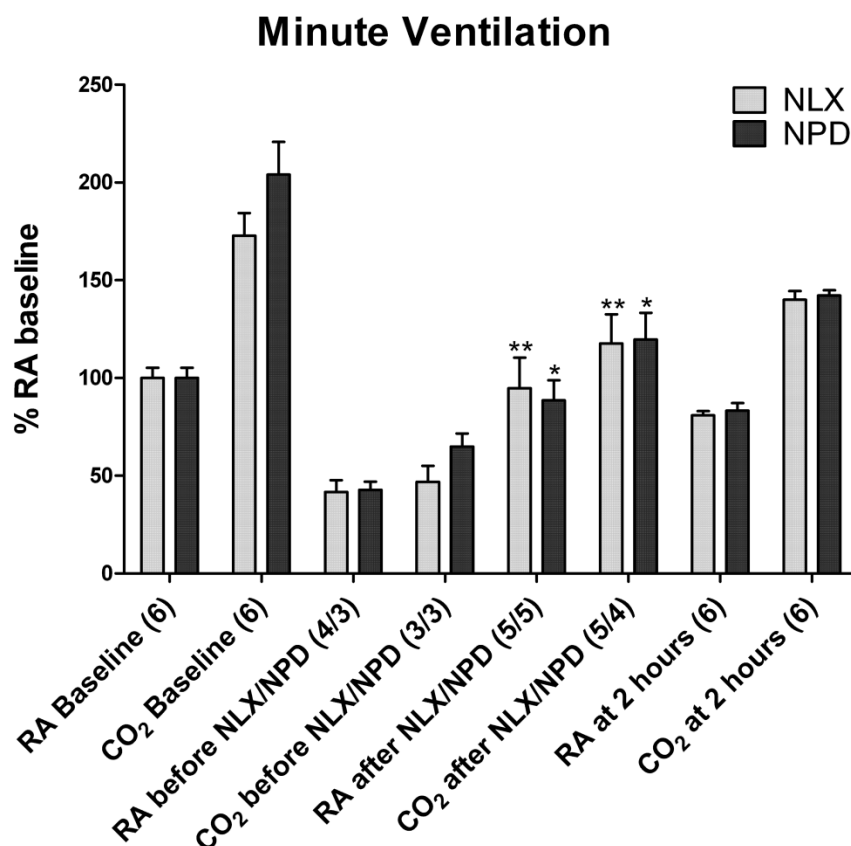


Figure 2a.5. Inspiratory minute ventilation was measured for animals receiving a 40 mg/kg morphine overdose followed by a NLX (grey bars) or NPD (black bars) rescue injection during severe morphine-induced respiratory depression. Data was normalized to RA baseline. V_I is reported at the beginning (baseline) and the end (at 2 hours) of the experiment. Measurements “before” and “after” refer to the time of the NLX or NPD rescue injection, respectively. Numbers indicate the number of animals for which data is available for the given time point. * $p < 0.05$, ** $p < 0.01$ vs RA or CO₂ before NLX/NPD

Figure 5 shows the minute ventilation data for both the NLX and NPD rescue studies.

Baseline measurements for both RA and CO₂ were not different from each other (**Table 2a.2**). Respiratory data before the injection represents the average of 1-2 min of either RA or CO₂ data that was recorded within the last 5 min prior to the rescue injection. Depending on the time of rescue or the criteria

met for the rescue, RA or CO₂ data is not available for all animals; the number of animals for all measurements is indicated in **Figure 2a.5**. Our pre-rescue injection V_I data shows no differences between the two groups, suggesting that comparable rescue criteria were employed for the NLX rescue or the NPD rescue. After rescue injection, NLX treated animals showed a strong recovery in less than a minute, while NPD injected animals recovered slower and showed a marked recovery after about 5 min. Our post rescue injection data shown in **Figure 2a.5** shows RA V_I for NLX at 5-10 min after and for NPD at 10-15 min after rescue. Similar for V_I during CO₂ challenge, NLX data represents 10-15 min after and NPD 15-25 min after rescue injection. This data suggests that the NPD rescue injection, though slower than and not as immediate as the free naloxone, can reverse severe respiratory depression caused by a 40 mg/kg morphine overdose. The decrease of respiratory depressive effects is not different in magnitude for both rescue injections, while the timeline of the rescue is. At two hours, the respiratory measurements for the rescue animals that had received the NLX or NPD after the morphine overdose were not different from those who had been co-injected.

Discussion

The purpose of this study was to test if a newly synthesized naloxone prodrug (Huang et al., 2009) can reverse the potentially life-threatening respiratory effects of an overdose of morphine *in vivo*. We developed a test protocol to safely study the respiratory depressive effects of a morphine overdose in Sprague-Dawley rats. Rats were administered an overdose of 40 mg/kg of morphine in combination with either a bolus injection of free naloxone or the newly synthesized naloxone prodrug containing an equal amount of naloxone. The present study focuses on the prevention and reversal of critical respiratory depression induced by a high dose of morphine. The chosen morphine overdose represents a 10-fold increase in morphine dose that causes life-threatening respiratory depression in a vast majority of test animals. As a control we used animals that were injected i.v. with vehicle (saline) or 4 mg/kg morphine, a dose that shows analgesia in our rat animal model, but no severe respiratory depression. In addition we studied the effects of both naloxone and naloxone prodrug alone.

For a two hour time period following a single i.v. tail vein injection, we monitored major respiratory parameters including inspiratory minute ventilation, frequency, tidal volume, inspiratory time, expiratory time, and inspiratory effort. Using a two chamber plethysmograph we were able to study the effects of the administered drugs without the confounding complications of additional restriction, sedation or anesthesia for the animal (Blake and Banchero 1985). Other than a rubber collar to separate the head chamber (where the breathing gases were administered) and the body chamber (where the respiratory measurements occurred via a pressure transducer), animals were confined to, but unrestricted in the testing apparatus, and awake during the monitoring period.

Our respiratory studies clearly suggest that the naloxone prodrug accomplishes the prevention of severe respiratory depression as needed, without presumably abolishing analgesia completely. At the tested ratio of 40:1 morphine to naloxone, the naloxone prodrug attenuated the respiratory effects to a level that is 1) safe and non-life-threatening and 2) comparable to the respiratory effects of an adequate analgesic morphine dose of 4 mg/kg in the rat. Importantly, this attenuation was present for the complete two hours of respiratory monitoring post injection. In contrast, at the same ratio of morphine to naloxone, a co-injection of a bolus dose of naloxone completely reverses the respiratory effects of the morphine overdose during the first hour, suggesting that at the same time the analgesic effects are reversed as well. The respiratory depressive effects of morphine return during the second hour after the effects of naloxone start to wear off. This demonstrates the problem of adequate dosing of the naloxone antagonist for it to be effective for a longer period of time. In clinical settings the short duration of naloxone is circumvented by monitoring the patient and repeatedly administering lower doses of naloxone (Takahashi et al., 2004). We found no respiratory depressive or stimulating effects of naloxone or the naloxone prodrug by themselves compared to our vehicle control, suggesting that all respiratory effects we see in our co-injected animals are a result of the reversal or prevention of the effects of the morphine overdose.

Our rescue study experiments clearly indicate that the overdose of 40 mg/kg morphine causes the predicted severe respiratory side-effects in almost all tested animals (92%), which without intervention would most likely have been detrimental. This dose was carefully chosen to model a severe overdose of morphine with potential lethal outcome (10-fold higher compared to an analgesic dose), while at the same time trying to avoid unnecessary harm and stress to the animals. As expected, the control rescue injection of free naloxone causes an almost immediate reversal of the depressive respiratory effects. The naloxone

prodrug is equally effective in rescuing the animals from severe respiratory depression but does so on a slower time-scale. Clearly, the naloxone prodrug would not be the antidote of choice to rescue a patient where immediate relief is needed, but these experiments show that the naloxone prodrug is capable of releasing naloxone quick enough to stabilize and slowly reverse respiratory depression that already existed prior to the prodrug administration.

Comparing the pharmacokinetic profile of free naloxone and naloxone released from the prodrug revealed differences in both the half-life time and the elimination constant. Both profiles were best fitted by first order kinetics of an i.v. bolus injection. Clearly though, the naloxone prodrug has some component of a release mechanism to its kinetic profile that can explain these differences, but is difficult to quantify due to the variability of each individual animal's response to the morphine overdose. In parallel to our rescue study, we expect that some animals have an early hypoxic episode within minutes after injection, while others reach severe respiratory depression later during the first hour. Given the profoundly different effects naloxone and the naloxone prodrug have on the respiratory effects of morphine, it is rather surprising the effects on the pharmacokinetic profile are not more significant. On the other hand, this certainly suggests that small changes in the naloxone concentration and pharmacodynamics can have significant consequences with regard to the respiratory changes they cause (Olofsen et al., 2010).

Interestingly, we showed that the presence of naloxone or naloxone prodrug has different effects on some of the pharmacokinetic parameters of morphine in the animal; specifically free naloxone has increased its clearance and decreased the extrapolated apparent concentration at time zero. Naloxone has been previously shown to reduce morphine concentration in the CNS and in plasma after a large dose of morphine, but not after a small dose of morphine (Miyamoto et al., 1993). The mechanisms for these changes are unclear, but it is speculated that changes are starting in the plasma through an increase in both morphine clearance and volume of distribution (Miyamoto et al., 1993), consistent with our observations where the presence of the free naloxone causes a significant increase in clearance and minor increase in the volume of distribution compared to the naloxone prodrug co-injected animals.

Adequate dosing of morphine can be seen as a feedback between the care provider and the patient. A new approach we are following here is to have the patient's body be its own feedback system. Our approach uses an agonist-antagonist based strategy, where the antagonistic component, the naloxone in the prodrug, is released as a feedback response. A characteristic of respiratory depression is hypoxia of tissue and in blood. We are using this as a trigger to release naloxone and counteract the respiratory effects as long as hypoxic conditions prevail. Our data suggests that the newly developed prodrug is inactive until a reductive environment in the blood stream caused by hypoxia is resulting in the release of naloxone. The exact degree of hypoxia for the naloxone release might vary from animal to animal and could not be determined in our study setup. Further studies in a larger animal model are needed to monitor the level of hypoxia required to activate the release of naloxone *in vivo*.

Task 3. Design and evaluate *in vitro* a Ketamine-based prodrug that yields 6 hours of pain relief following a single administration

Permissions to perform Ketamine work is in a regulatory stalemate.

The DEA license of Dr. Vanderspek was usurped by the University of Michigan Medical Center as an educational license. This restricts Dr. Vanderspek's work to clinical care in the clinical buildings of the University of Michigan only; specifically, research cannot be conducted on that license. The University of Michigan is willing to release the license once it is up for renewal in March 2013; efforts to release it before that time have so far failed. The DEA has stipulated that it will not issue a separate research license for Dr. Vanderspek and requires him to add each building in which research is conducted for the DARPA project. Unfortunately, Dr. Vanderspek's DEA license, which is controlled by the University of Michigan,, does not allow addition of multiple sites. Additionally, Hospira has decided to not provide (sell, grant or otherwise) pure ketamine for this project. This means that if or when licensing issues have been resolved, commercially available, clinically formulated ketamine will have to be purified for use in this project.

Task 4. Design and evaluate *in vitro* an Atropine-Pralidoxime-Extended-Release-Therapeutic (APERT) capable of sustained release for up to 12-24 hours.

Summary. We have investigated the dendrimer-enabled delivery platform that is designed to develop more effective therapeutic methods for the treatment and prophylaxis of organophosphate (OP) poisoning. This platform aims to release antidote small molecules in a sustained and feedback-based mechanism. It is based on a fifth generation (G5) polyamidoamine (PAMAM) dendrimer designed with multifunctional features that display three critical functions (**Figure 4.1**). First, it serves a drug carrier by providing drug-binding

cavities for pralidoxime (2-PAM) molecules, and slows extended duration of drug action through a sustained release mechanism.

Second, the carrier itself displays a

built-in therapeutic activity as the catalytic scavenger of OP agents. Third, the dendrimer is thus designed to sense OP agents and to release its 2-PAM payloads in response to such OP exposure.

In this year, our main achievement is three-fold. First, we demonstrated that the dendrimer platform was more effective than 2-PAM in destroying an OP agent in a physiological condition (plasma, 37°C). Second, we optimized an AChE reactivation assay, and started screening of the dendrimer carriers. Third, we have developed a laboratory protocol for an *in vivo* rescue study in a mouse model using paraoxon (POX) as a model OP.

4.1. Multi-gram scale up synthesis of dendrimer-oxime conjugates

For scale synthesis, we selected two drug-conjugated dendrimers G5-GHA, and G5-(4PAM), as shown in **Figure 4.2**. Each dendrimer is conjugated with multiple copies of antidote oxime molecules, hydroxamate and 4-PAM, each attached covalently to the dendrimer surface. Our earlier study demonstrated that each of these conjugates display

potent OP scavenging activities. The synthetic process starts with an EDC-based activation process for both G5-GA and G5-EG. For G5-GHA synthesis, the activated dendrimer then reacted with hydroxylamine, by which 1.5g of G5-GHA was obtained. The other conjugate G5-4PAM was prepared

Figure 4.1 Design of a dendrimer drug carrier, and its proposed mechanism of action that releases its 2-PAM payloads in response to OP nerve agents. Red dot: 2-PAM conjugated (covalently attached); Blue dot: 2-PAM complexed (non-covalent)

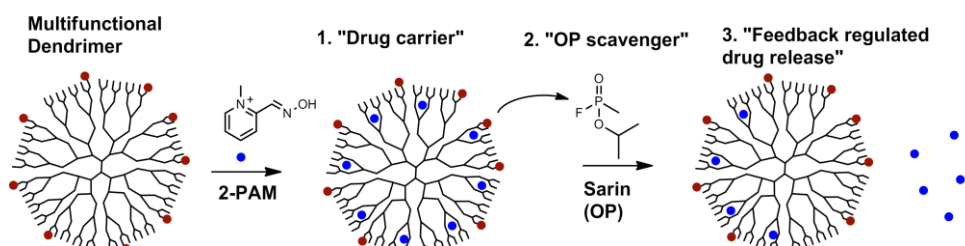
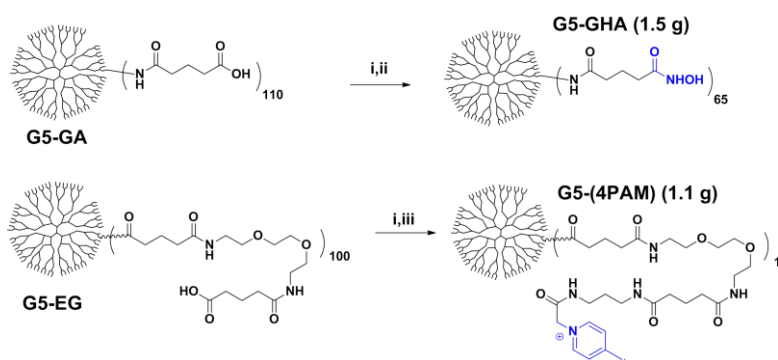


Figure 4.2 Gram scale synthesis of two dendrimer-drug conjugates, each conjugated with hydroxamate or 4-PAM antidote molecules.



in 1.1 g in a similar manner by reacting with 4-PAM following the activation step. Purification of these dendrimer conjugates was performed by the membrane dialysis that led to greater than 95% purity. Advantages of this synthetic process include such a simple purification method, and short synthetic steps, combination of which allowed rapid and multi-gram synthesis of the conjugates. In summary, we developed a two-step divergent synthetic method for conjugation of oxime drug molecules to the G5 PAMAM dendrimer.

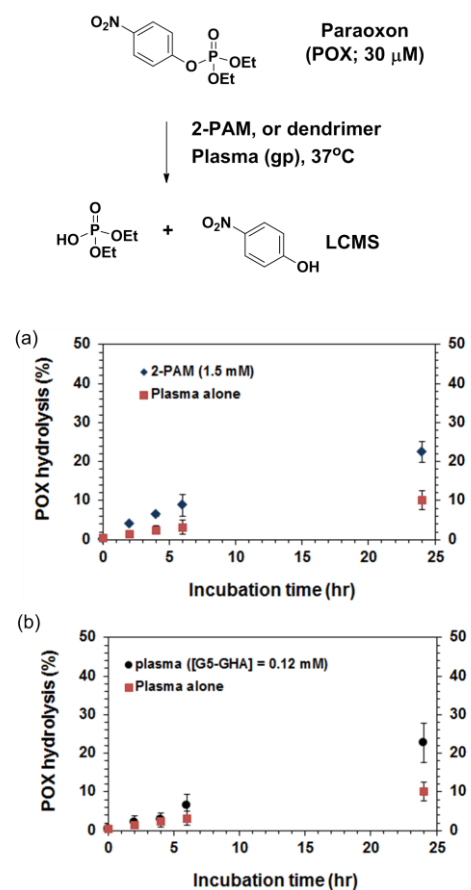
4.2. POX scavenging activity in a physiological condition

We investigated OP scavenging activities by drug-conjugated dendrimers in plasma (guinea pig) at 37°C using paraoxon (POX; 30 μ M) as an OP agent (**Figure 4.3**). Reaction kinetics of POX hydrolysis was monitored by LCMS/MS spectrometry in which 4-nitrophenol (4-NP), the byproduct of POX hydrolysis, was quantified. In this bioanalytical assay, we first generated standard mass spec calibration curves of 4-NP, and developed a protocol for processing each plasma sample in a quality optimized for mass spec determination of 4-NP concentrations.

Figure 4.3 illustrates the kinetics of POX hydrolysis studied by this mass spec method. Plasma alone displayed a basal activity at the level of about 10% after 24 h incubation. This activity is not due to simple chemical hydrolysis of POX because POX was quite stable (<1% hydrolysis) in PBS (pH 7.4, 37°C; not shown). Therefore such POX hydrolysis is attributed to enzymatic hydrolysis possibly by serum proteins including phosphatases and paraoxonase (PON). 2-PAM (1.5 mM) facilitated the POX hydrolysis to a greater extent and in a time dependent manner. About 23% of POX was hydrolyzed by 2-PAM after 24 h, a net 13% increase relative to the plasma alone. We also observed a similar or slightly greater activity by G5-GHA (0.12 mM) even at a lower dose (0.12 mM).

This study confirms another mode of action by 2-PAM as an OP scavenger. Despite its OP scavenging activity observed *in vitro*, 2-PAM cannot make such significant activity *in vivo* due to its short plasma half-life (1–1.5 h). However, it is highly encouraging to see the same level of POX scavenging activity by G5-GHA conjugate which should have an extended duration of circulation (~24 h).

Figure 4.3 POX hydrolysis by 2-PAM (a) or a dendrimer conjugate G5-GHA (b), each studied in guinea pig plasma at 37°C.



4.3. Rate of POX hydrolysis

We investigated reaction kinetics of POX hydrolysis catalyzed by oxime drugs and dendrimer-drug conjugates. It was performed in PBS (pH 7.4) instead of plasma because the latter complicates the kinetic analysis by its inherent protein activity. We employed a colorimetric UV-vis assay in order to monitor 4-nitrophenol on a real time basis. Figure 4 shows the POX hydrolysis catalyzed by three oxime drugs and three different dendrimer conjugates. The magnitude of the catalytic activity is expressed in terms of half-life ($t_{1/2}$) which is defined as the time taken to reach 50% hydrolysis of POX. **Figure 4.4a** illustrates that each oxime compound facilitated POX hydrolysis compared to the buffer control. It also shows the temperature effect, indicating that POX hydrolysis occurs faster at 37°C than rt. Among three oxime drugs, homodimeric oximes, obidoxime and trimedoxime, had 2- to 3-fold greater activity than 2-PAM at a given temperature. Such difference might be accounted for by total concentrations of oxime functionality which involves in the catalytic reaction ($[\text{oxime}]_{2\text{-PAM}} = [2\text{-PAM}]$; $[\text{oxime}]_{\text{homodimer}} = 2 \times [\text{obidoxime or trimedoxime}]$). Figure 4b shows the catalytic activities displayed by three dendrimer-drug conjugates. All of them facilitated POX hydrolysis similarly or greater than 2-PAM. Notably, the G5-GHA conjugate ($t_{1/2} \approx 6$ h) facilitated POX hydrolysis ~5-fold faster than 2-PAM ($t_{1/2} \approx 35$ h).

4.3. Quantitative analysis of rate constants of POX hydrolysis

After OP scavenging activity observed by drug-conjugated dendrimers, we further studied kinetic parameters associated with the mechanism of POX hydrolysis. **Figure 4.5** describes the two-step mechanism of POX hydrolysis catalyzed by an oxime drug molecule either free or as a conjugated form on the dendrimer surface. It comprises of two steps: i) a nucleophilic attack of an oxime molecule at POX which constitutes a slow, rate-determining step (k_1); ii) hydrolysis of the resultant oxime-OP adduct which occurs much faster (k_2). Thus the reaction rate of POX hydrolysis is determined primarily at the first step. The k_1 rate constants determined for three oxime drugs indicate that they are almost identical in the activity: k_1 ($\text{M}^{-1}\text{min}^{-1}$) = 0.21 (2-PAM, obidoxime), 0.23 (trimedoxime).

Figure 4.4 (a) Temperature effect on hydrolysis of POX (30 μM) catalyzed by oxime drugs in PBS ([drug] = 1.5 mM; pH 7.4); (b) Comparison of half lives of hydrolysis at 37°C between oxime drugs (each 1.5 mM) and dendrimer-drug conjugates (each 0.12 mM).

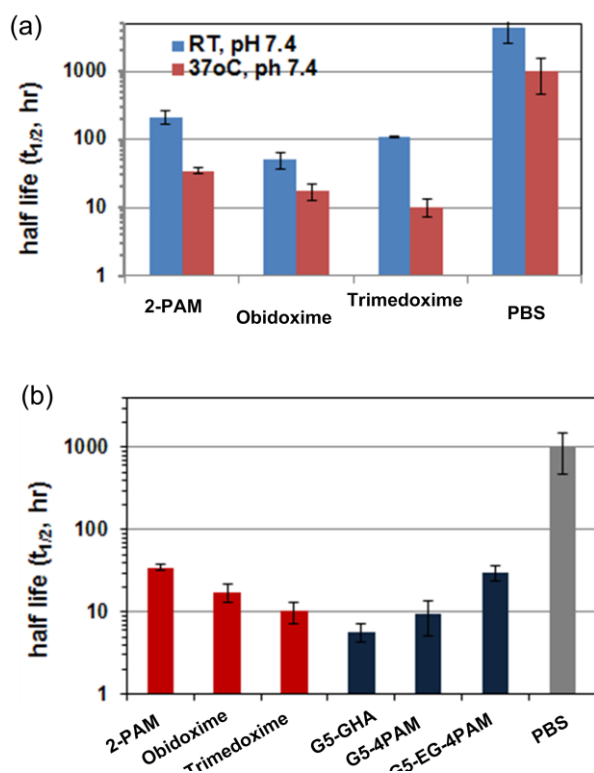
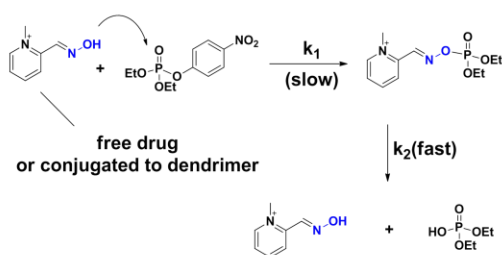


Figure 4.5 Summary of rate constant values (k_1) for rate-determining step of POX hydrolysis (pH 7.4, 37°C)



Oxime, or dendrimer conjugate	k_1 ($M^{-1}min^{-1}$)
2-PAM	0.21
Obidoxime	0.21
Trimedoxime	0.23
G5-GHA	0.14
G5-(4PAM)	0.56

We then investigated if drug conjugation to the dendrimer makes an effect on the first step. The k_1 values were determined for two selected dendrimer-drug conjugates, G5-GHA, and G5-(4PAM). G5-GHA shows a slightly lowered but comparable rate constant (0.14) to free 2-PAM and obidoxime (0.21). The other conjugate G5-(4PAM) showed a greater rate constant ($k_1 = 0.56$). This mechanistic study suggests that an oxime drug molecule conjugated to the dendrimer surface still retains or even improves the POX scavenging activity.

4.4. AChE reactivation assay

We have standardized the assay for oxime reactivation of AChE, using the Ellman's assay as described previously (Kalisiak et al, *J Med Chem*, 54: 3319-3330, 2011). As shown in **Figure 4.6**, 2-PAM reactivated POX-inhibited AChE in a time dependent manner, with ~80% inhibition by 100 nM POX, which was reactivated by 100 μ M 2-PAM to reach ~80% of the control enzyme activity. We observed similar reactivation by the oximes obidoxime and trimedoxime, although with these agents complete

reactivation was achieved in <15 min. In our preliminary studies, we observed modest reactivation by the synthesized G5-GHA (**Figure 4.7**). Further studies are needed to evaluate reactivation of different dendrimer-oxime conjugates. If we fail to observe significant reactivation of stable amide coupled oximes, we will synthesize compounds with ester-linked oximes which would allow the slow release of free oximes for AChE reactivation.

In summary, we designed a novel class of dendrimer-oxime drug conjugates, and evaluated their therapeutic potential for the treatment of OP poisoning. Each of these dendrimer conjugates is tethered on its surface with oxime drug (or drug-equivalent) molecules. It actively catalyzed OP hydrolysis as demonstrated by using the POX model. We further determined kinetics and mechanism of POX hydrolysis by the conjugates, and developed efficient large scale synthetic methods for two conjugates (G5-GHA; G5-4PAM). Future studies will focus on their efficacy in a mouse model.

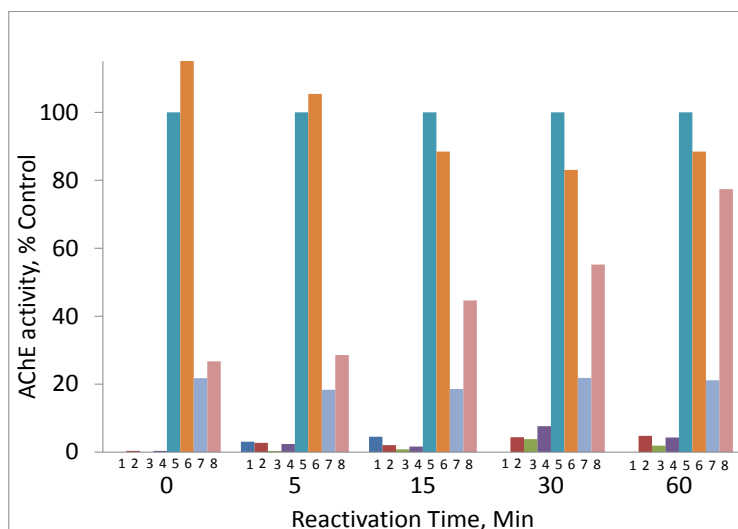


Figure 4.6| Time dependent reactivation of acetyl choline esterase (AChE) by 2-PAM. ~4.5 U/ml of purified human erythrocyte AChE (Sigma) in PBSL (PBS containing 50 μ g/ml β -lactoglobulin) was incubated with 100 nM POX for 15 min at 37°C, and filtered using a 10 KDa MWCO filter to remove unbound POX. Control and POX treated AChE was then incubated with 100 μ M 2-PAM for 1 h at RT. The incubation mixture was diluted 20 x fold in PBSL and the activity of AChE was determined by Ellmans assay. For each time points, the bars represent (from left to right), 1. Control AChE in the absence of the substrate acetyl thiocholine; 2. Pox-treated AChE in the absence of the substrate acetyl thiocholine; 3. Blank in the absence of any AChE; 4. Effect of 2-PAM in the absence of any enzyme (oximolysis); 5. Control AChE activity; 6. Control AChE plus 2-PAM; 7. Activity of POX-treated AChE; 8. Reactivation of POX-treated AChE by 2-PAM. The data is represented as the percentage of the activity of control AChE (Bar 5).

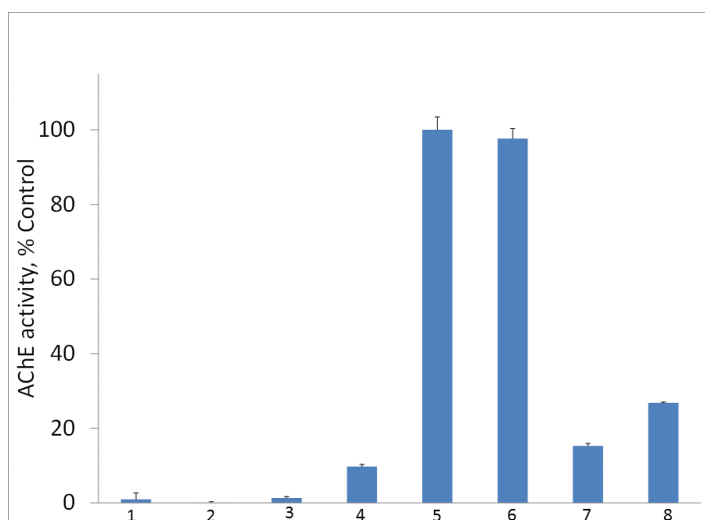


Figure 4.7| Reactivation of AChE by G5-GHA (1 h reactivation). The assay conditions and the identity of the bars 1 through 8 are similar to that described under the legend for Figure 4.6.

Intellectual Property Generated During Contract

Invention Title: **UM 3709 – Dendrimeric Prodrug as a Controlled Release Formulation in Pain Management – Patent Title: Dendrimer Conjugates**

Patent/Application Numbers: **61/101,461; 12/570,977**

Is the Patent Filed in the US?: **Yes**

Is the Patent Filed in a Foreign Country?: **No**

Was the confirmatory instrument or assignment forwarded to the contracting officer?: **Yes**

Foreign Countries of application: **None**

Invention Title: **UM 4538 Synthesis of Baker-Huang PAMAM Dendrimers; Patent Title Dendrimer Compositions and Methods of Synthesis**

Patent/Application Numbers: 61/251,244 and **13/502,004 (US), 2010318637 (AU), PCT/US2010/051835 (CN), 2777682 (CA)**

Is the Patent Filed in the US?: **Yes**

Is the Patent Filed in a Foreign Country?: **Yes**

Was the confirmatory instrument or assignment forwarded to the contracting officer?: **Yes**

Foreign Countries of application: **China, Canada, Australia**

Invention Title: **UM 4603 – Block Synthetic Method for Dendrimer Synthesis**

Patent/Application Numbers: **Not filed**

Is the Patent Filed in the US?: **No**

Is the Patent Filed in a Foreign Country?: **No**

Was the confirmatory instrument or assignment forwarded to the contracting officer?: **Yes**

Foreign Countries of application: **No**

Invention Title: **UM 4798 – Multifunctional Small Molecules**

Patent/Application Numbers: **13/504,046**

Is the Patent Filed in the US?: **Yes**

Is the Patent Filed in a Foreign Country?: **No**

Was the confirmatory instrument or assignment forwarded to the contracting officer?: **Yes**

Foreign Countries of application: **No**

Invention Title: **UM 4901 – Dual Function Real-Time Sensor**

Patent/Application Numbers: **Per legal counsel not filed**

Is the Patent Filed in the US?: **No**

Is the Patent Filed in a Foreign Country?: **No**

Was the confirmatory instrument or assignment forwarded to the contracting officer?: **Yes**

Foreign Countries of application: **None**

Invention Title: **UM 4902 – Bioorthogonal Nanoparticle Reporter Alkyne System**

Patent/Application Numbers: **61/562,767**

Is the Patent Filed in the US?: **Yes; Synthesizing Functionalized Dendrimers within Biological Settings**

Is the Patent Filed in a Foreign Country?: **No**

Was the confirmatory instrument or assignment forwarded to the contracting officer?: **Yes**

Foreign Countries of application: **None**

Invention Title: **UM 4966 – Plasmin Release**

Patent/Application Numbers: **None**

Is the Patent Filed in the US?: **No**

Is the Patent Filed in a Foreign Country?: **No**

Was the confirmatory instrument or assignment forwarded to the contracting officer?: **Yes**

Foreign Countries of application: **None**

Invention Title: **UM 5132 – Dendrimer Conjugate and Methotrexate Analogues**

Patent/Application Numbers: **61/568,521(provisional) – Multifunctional Small molecules**

Is the Patent Filed in the US?: **Yes**

Is the Patent Filed in a Foreign Country?: **No**

Was the confirmatory instrument or assignment forwarded to the contracting officer?: **Yes**

Foreign Countries of application: **None**

Invention Title: **UM 5186 – Organophosphate Antidotes**

Patent/Application Numbers: **61/625,911(provisional) – Multifunctional Small molecules**

Is the Patent Filed in the US?: **Yes**

Is the Patent Filed in a Foreign Country?: **No**

Was the confirmatory instrument or assignment forwarded to the contracting officer?: **Yes**

Foreign Countries of application: **Not determined at this point in time**

Invention Title: **UM 5217 – Antibacterial Dendrimer**

Patent/Application Numbers: **Not filed**

Is the Patent Filed in the US?:

Is the Patent Filed in a Foreign Country?:

Was the confirmatory instrument or assignment forwarded to the contracting officer?: **Yes**

Foreign Countries of application: **No**

Invention Title: **UM 5608 – Fluorescent Dye Conjugate**

Patent/Application Numbers: **Not filed**

Is the Patent Filed in the US?:

Is the Patent Filed in a Foreign Country?:

Was the confirmatory instrument or assignment forwarded to the contracting officer?: **Yes**

Foreign Countries of application: **No**

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